



The effects of a CXCR1/CXCR2 antagonist on neutrophil migration in mild atopic asthmatic subjects



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ARTICLE INFO

Article history:

Received 5 December 2015

Received in revised form

1 September 2016

Accepted 13 September 2016

Available online 14 September 2016

Keywords:

CXCR2
Neutrophil migration
Peripheral blood
Bone marrow
Induced sputum
Asthma

ABSTRACT

Background: Neutrophils are effector cells recruited to airways in patients with asthma. Migration of neutrophils occurs predominantly through activation of the CXCR1 and CXCR2 receptors by CXC chemokines, including IL-8 and Gro- α . The dual CXCR1/CXCR2 antagonist SCH 527123 has been developed to target neutrophil migration to alleviate airway neutrophilia. This study investigated the effects of SCH 527123 on neutrophil levels within the bone marrow, peripheral blood and airways, and on isolated bone marrow and peripheral blood neutrophil migration from mild allergic asthmatics.

Methods: Thirteen subjects with mild allergic asthma completed a double blind, placebo-controlled, multi-center crossover study and were randomized to daily dosing of 30 mg SCH 527123 and placebo for 8 days. Subjects provided bone marrow, peripheral blood and sputum samples pre-dosing and on the last day of dosing. Neutrophil numbers were quantified in all samples and chemotaxis assays were performed on neutrophils purified from bone marrow and peripheral blood.

Results: Neutrophil numbers fell significantly in the peripheral blood and sputum following treatment with SCH 527123 compared to placebo treatment. No change in neutrophil numbers was observed in bone marrow. SCH 527123 reduced IL-8-induced migration of purified peripheral blood neutrophils ($p < 0.05$), but had limited effects on migration of neutrophils purified from bone marrow.

Conclusions: The results from this study demonstrate that oral administration of the dual CXCR1/CXCR2 antagonist SCH 527123 reduces neutrophil levels in the circulation and airways through inhibition of migration. There were no toxic effects of SCH 527123 on granulocytic progenitor cells in the bone marrow.

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1. Introduction

There is a growing appreciation that asthma is a heterogeneous

Abbreviations: FEV₁, Forced Expiratory Volume in 1 s; BM, Bone Marrow; PB, Peripheral Blood; IS, Induced Sputum; SAE, Serious Adverse Events; SCH 527123, 2-hydroxy-N,N-dimethyl-3-[(2-[(R)-1-(5-methyl-furan-2-yl)-propyl]amino)-3,4-dioxo-cyclobut-1-enylamino]-benzamide; HBSS, Hanks Balanced Salt Solution; LPS, Lipopolysaccharide; BAL, Bronchoalveolar Lavage; CXCL12, Stromal derived factor 1; CXCR2, CXC Chemokine Receptor 2; fMLP, formyl-methionyl-leucyl-phenylalanine.

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<http://dx.doi.org/10.1016/j.ypupt.2016.09.005>

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condition with multiple potential phenotypes and differing inflammatory profiles [1]. Neutrophilic airway inflammation is a distinct inflammatory process that may be present either alone or in conjunction with eosinophilic inflammation [2], and is typically associated with a severe asthma phenotype [3], which often does not respond well to steroid treatment [4].

Neutrophils are recruited to the lung in response to chemokines such as IL-8, found at high levels in the airways of asthmatics [5,6]. The biological effects of IL-8 are mediated through the chemokine receptors CXCR1 and CXCR2, stimulation of which induces neutrophil chemotaxis [7–9]. CXCL1 (Gro- α) is a chemokine structurally related to IL-8, and selectively activates CXCR2 [10] to promote neutrophil inflammation [11]. CXCR2 has been thought to

be involved in uncontrolled neutrophil influx into the airways in acute lung injury [12]. Deletion of TLR4 abolishes CXCL chemokine secretion, thereby inhibiting neutrophil recruitment to the airways induced by a ragweed pollen extract challenge in mice [13]. On the other hand, intratracheal administration of IL-33 induces exacerbated neutrophilic inflammation and AHR associated with increased CXCL1 and CXCL2; subsequent treatment with anti-CXCR2 repressed neutrophilic inflammation and AHR, suggesting that IL-33 can enhance neutrophilic inflammation via CXCR2 signaling [14]. In cases of viral infection, the human rhinovirus has been shown to have neutrophilic inflammation as a key component, which is dependent on CXCR2 signaling [15]. These findings suggest that neutrophil lung-homing is CXCR2-dependent, and is a critical rate-limiting event that stimulates AHR and airway inflammation. Interestingly, the expression of both CXCR1 and CXCR2 has been shown to increase following treatment with β -agonists and corticosteroid, without influencing neutrophil lung-homing [16]. CXCR2 has further been identified as a biomarker that is higher in the sputum of patients with neutrophilic asthma [17].

The recognition of the pivotal roles of CXCR1 and CXCR2 in the pathogenesis of neutrophilic inflammation has prompted the development of several small molecule antagonists targeting these receptors [7]. For example, oral administration of SB-656933 has previously been shown to inhibit increased neutrophil numbers in sputum as a result of ozone challenges in normal volunteers [18]. Furthermore, oral administration of the CXCR2 antagonist AZD8309 significantly inhibited LPS-induced inflammation measured in sputum of normal volunteers [19]. SCH 527123 (also known as MK-7123) is a novel small molecule that is a potent and selective allosteric antagonist of the human CXCR1 and CXCR2 receptors [20]. SCH 527123 inhibits neutrophil trafficking to ELR-CXC chemokines (IL-8, Gro- α , ENA-78, NAP-2). Oral treatment with SCH 527123 in healthy human subjects significantly inhibits ozone-induced airway neutrophilia, IL-8 and MPO levels within sputum [21], and in patients with severe asthma it reduced sputum neutrophils and mild exacerbations [22].

The aim of this study was to examine the mechanisms by which oral treatment with SCH 527123 can effect neutrophil migration in subjects with mild allergic asthma.

2. Methods

2.1. Subjects

Nineteen non-smoking subjects between the ages of 19–48 years old were randomized, and thirteen subjects completed the study (Table 1, Fig. 1). The sample size was based on feasibility, and selected to allow at least 12 subjects to complete all assessments. No formal power calculations could be performed for the exploratory outcomes in this study. Seven of the 13 subjects provided a complete set of sputum samples for a sub-study to evaluate the function of neutrophils in blood and bone marrow using an *in vitro* assay. The study was approved by the Research Ethics Committees at McMaster University and Institut Universitaire de Cardiologie et

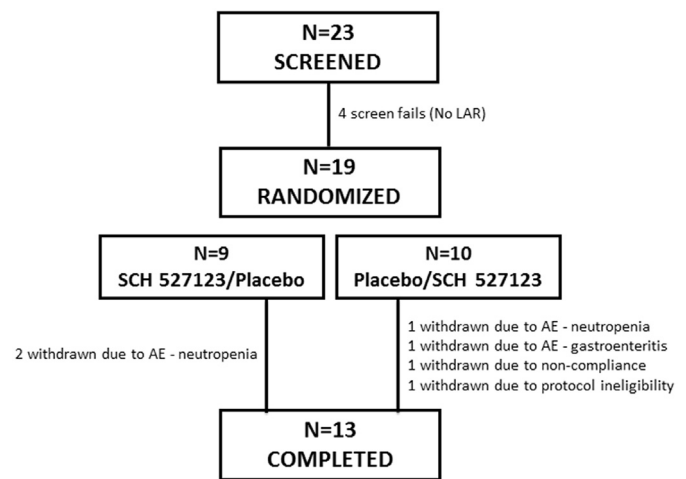


Fig. 1. Consort diagram.

de Pneumologie de Québec, Laval University, and all subjects provided written informed consent.

Subjects were required to have mild allergic asthma with methacholine PC₂₀ $\leq$ 16 mg/mL, FEV₁ $\geq$ 70% of predicted, free from asthma exacerbation for at least 4 weeks, and using only inhaled short-acting β_2 -agonists less than twice weekly for treatment of asthma. All subjects tested positive to common aeroallergens by skin prick test to common allergens. Subjects were excluded if they have been diagnosed with any other lung disease, or had a peripheral blood neutrophil count of $< 3 \times 10^6$ /mL. Subjects were discontinued from the trial if the peripheral blood neutrophil count dropped below 1.5×10^6 /mL.

2.2. Study design

The study was a randomized, double blind, placebo-controlled, multi-center, crossover trial to evaluate the effects of SCH 527123 on the level of neutrophils in the bone marrow, peripheral blood, and induced sputum of subjects with mild allergic asthma. Subjects meeting the study criteria were randomized to receive 30 mg SCH 527123 or placebo administered orally, once daily for 8 days. This dose was selected to maintain neutrophil levels above a threshold of 1.5×10^6 /mL for the duration of the study. Treatment periods were separated by 2–4 weeks (Fig. 2).

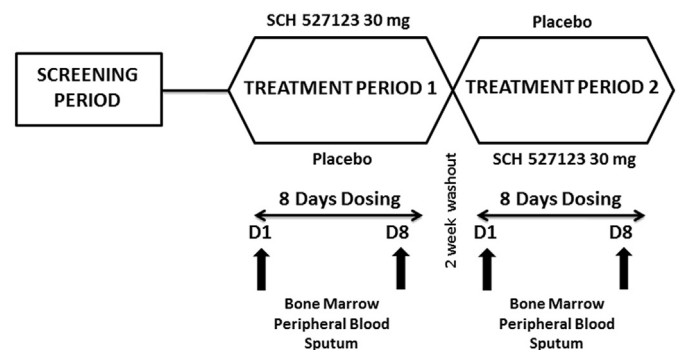


Fig. 2. Study Design Schematic. Randomized, double-blind, placebo-controlled crossover trial comparing the effects of 30 mg SCH 527123 once daily for 8 days, to placebo. Samples of bone marrow, peripheral blood, and induced sputum were obtained pre-dosing on Day 1, and post-dosing on Day 8 of each treatment period.

Table 1

Subject characteristics are shown as mean $\pm$ SEM. Methacholine PC₂₀ is shown as geometric mean and range. FEV₁: Forced expiratory volume in 1 s. PC₂₀: Provocative concentration of methacholine causing 20% fall in FEV₁.

Male/Female	6/13
Age (years)	26.8 $\pm$ 2.2
FEV ₁ (% of predicted)	89.5 $\pm$ 0.2
Methacholine PC ₂₀ (mg/mL)	1.9 (0.07–10.9)

2.3. Randomization and dosing

Randomization was performed using computer-generated codes and kits were assigned based on the next number available. Drug and placebo capsules were identical in appearance and supplied by Schering-Plough (Merck & Co., Inc., Kenilworth, NJ, USA).

2.4. Sample collection

Venous blood was collected into sodium heparin vacutainers. Bone marrow from the anterior iliac crest was aspirated into a 10 mL syringe containing 1 mL sterile heparin. Peripheral blood samples were assessed total cell count and differentials, and investigators remained blinded to these results during the study. Bone marrow cell counts were obtained using a hemocytometer, and two bone marrow smears were prepared on glass slides, stained using Diff Quik, and a partial differential including mature neutrophils, immature neutrophils was performed at the end of the study. Sputum samples were induced using hypertonic saline inhalation and mucus plugs were selected and processed with 0.1% dithiothreitol, as previously described [23]. Cytospins of sputum cells were prepared on glass slides and stained with Diff Quik for a differential count expressed as the percentage of non-squamous cells.

2.5. Neutrophil chemotaxis assay in mild asthmatics

Peripheral blood and bone marrow samples were diluted in HBSS and granulocytes were purified with an Accuprep density gradient followed by lysis of erythrocytes. 50 μ L of granulocytes at a concentration of 300,000/well were loaded into a modified 96-well Boyden Chamber (Neuroprobe Inc, MD, USA) with a 3 μ m pore polycarbonate filter and incubated for 1 h in a 5% CO₂ incubator at 37 °C to assess chemotactic activity to IL-8. The number of cells migrating through the filter was quantified with MTS CellTiter 96 AqueousOne Solution Reagent by measuring absorbance at 490 nm

in an ELISA plate reader. Chemotaxis to 100 ng/mL of IL-8 (ie: CXCR2-mediated) and 10⁻⁸ M fMLP (ie: not CXCR2-mediated) was assessed in neutrophils purified from blood and bone marrow collected pre and 8 days post-treatment. These cells were pre-incubated for 15 min with 10⁻⁴ M SCH 527123 or diluent.

2.6. Statistical analysis

Summary data for all completed subjects are shown as mean $\pm$ SEM, and analyzed by ANOVA (chemotaxis assays) and paired t-tests (blood, bone marrow and sputum cell counts). A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Safety

SCH 527123 was safe and well tolerated. Neutropenia (peripheral blood neutrophil levels < 1.5 $\times$ 10⁶/mL) related to the mechanism of action of SCH 527123 was reported by 3 of the 19 subjects. Except for neutropenia, the occurrence of adverse events was similar between SCH 527123 and placebo.

3.2. Peripheral blood

Peripheral blood neutrophil levels dropped below 1.5 $\times$ 10⁶/mL in 4 subjects; three of these subjects were discontinued from the study. In the subjects that completed the study, eight days of oral administration with 30 mg SCH527123 significantly reduced the number of peripheral blood neutrophils from 3.5 $\pm$ 0.3 $\times$ 10⁶/mL pre-dosing to 3.0 $\pm$ 0.3 $\times$ 10⁶/mL post-treatment, and with placebo treatment there was no change in neutrophil numbers from 3.7 $\pm$ 0.3 $\times$ 10⁶/mL pre-dosing to 3.7 $\pm$ 0.3 $\times$ 10⁶/mL after 8 days (Fig. 3 p = 0.04) There was no effect of SCH 527123 on eosinophil numbers (p > 0.05) (Fig. 3C), or other leukocyte levels in peripheral blood (data not shown).

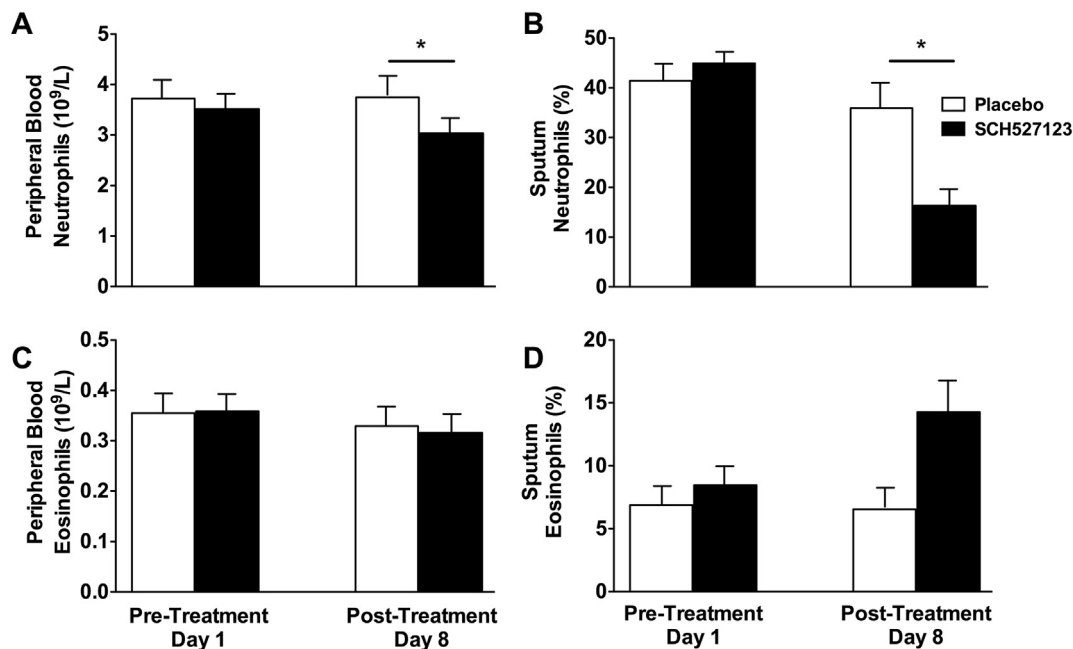


Fig. 3. Blood and sputum neutrophil and eosinophil counts pre- and post-treatment with placebo and SCH 527123. The effect of 30 mg SCH 527123 (solid bars) compared to placebo (open bars) once daily for 8 days on the number of neutrophils and eosinophils in peripheral, and on the percentage of neutrophils and eosinophils in sputum at pre-dosing on Day 1 and post-dosing on Day 8. Data are shown as mean $\pm$ SEM. *p < 0.05 compared to placebo.

3.3. Induced sputum

Differential cell counts were analyzed from induced sputum collected from a subgroup of 7 subjects who provided samples at each time point. Sputum neutrophils decreased from $44.9 \pm 1.7\%$ pre-dosing to $16.3 \pm 0.9\%$ after SCH 527123 and this decrease was significantly greater than placebo, being $41.4 \pm 1.7\%$ pre-dosing to $35.9 \pm 2.2\%$ after placebo treatment ($p < 0.05$) (Fig. 3). There was no significant effect of SCH 527123 on the level of other cells including eosinophils (Fig. 3), macrophages or lymphocytes (data not shown) ($p > 0.05$).

3.4. Bone marrow

There was no change in the number ($\times 10^6$ cells/mL) or percentage of neutrophils in the bone marrow after treatment with SCH 527123 or placebo ($p > 0.05$) (Fig. 4).

3.5. Neutrophil chemotaxis

Neutrophil migration was optimal with 100 ng/mL of IL-8 and 10^{-8} M fMLP versus no effect with diluent control (data not shown). These optimal concentrations of IL-8 and fMLP were used for the subsequent chemotaxis assays conducted on neutrophils purified from bone marrow and peripheral blood collected before and after 8 days of dosing with SCH 527123 and placebo, and compared to diluent control. Neutrophils purified from peripheral blood migrated consistently to the chemoattractants IL-8 and fMLP compared to diluent control ($p < 0.05$). On the other hand, neutrophils purified from bone marrow were less responsive to both IL-8 and fMLP (Fig. 5), showing significant migration compared to diluent control on only one of the four days (day 1 placebo).

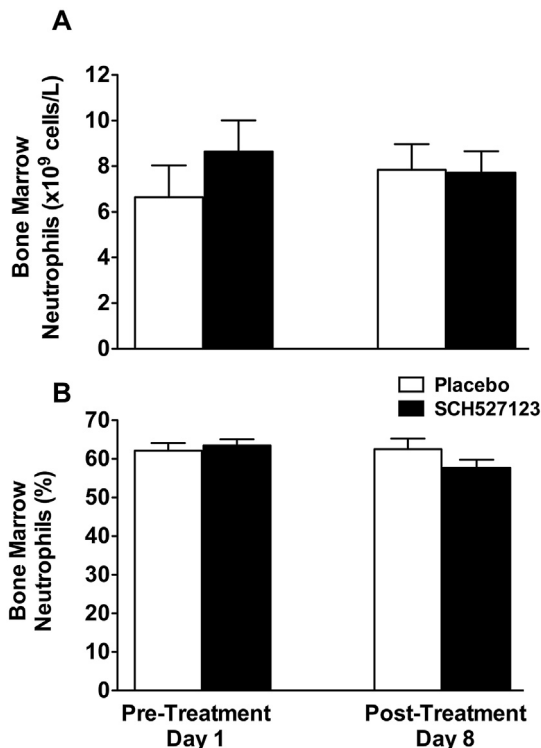


Fig. 4. Bone marrow neutrophil counts pre- and post-treatment with placebo and SCH 527123. The effect of 30 mg SCH 527123 (solid bars) compared to placebo (open bars) once daily for 8 days on the mean neutrophil (A) absolute cell count (B) percentages measured in the bone marrow at pre-dosing on Day 1 and post-dosing on Day 8. Data are shown as mean $\pm$ SEM. * $p < 0.05$ compared to placebo.

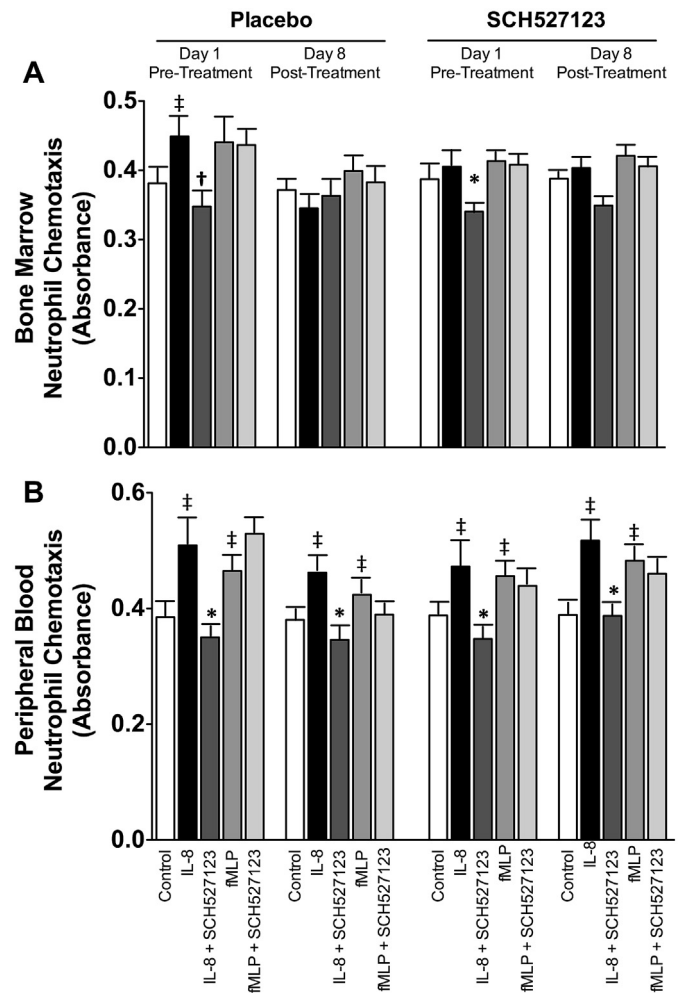


Fig. 5. SCH 527123 treatment inhibits IL-8-induced neutrophil chemotaxis. The effect of pre-incubating neutrophils purified from (A) bone marrow and (B) peripheral blood with SCH 527123, on IL-8-induced and fMLP-induced chemotaxis. Samples were collected from subjects during placebo (left) and SCH 527123 (right) treatment periods on Day 1 pre-dosing and Day 8 post-dosing. Data are expressed as mean $\pm$ SEM. ‡ $p < 0.05$ compared to PBS-stimulated control; * $p < 0.05$ compared to IL-8 stimulation; † $p < 0.10$ compared to IL-8 stimulation.

To evaluate the effects of *in vivo* oral dosing of SCH 527123 on neutrophil function, several controls were applied. In one of the experimental conditions, neutrophils were pre-incubated *in vitro* with 1.0×10^{-4} M SCH 527123 as a positive control; this consistently attenuated IL-8-induced migration of peripheral blood neutrophils on all study days ($p = 0.023$), but only attenuated migration of bone marrow neutrophils on the pre-dosing days ($p = 0.002$ day 1 SCH 527123-treatment, $p = 0.07$ day 1 placebo-treatment) (Fig. 5). Another of the experimental conditions measured migration to fMLP to confirm specificity of the effects of SCH 527123 through CXCR1/CXCR2. As expected, pre-treatment of neutrophils *in vitro* with 1×10^{-4} M SCH 527123 had no effect on fMLP-induced migration (Fig. 5).

When examining the effects of *in vivo* oral dosing of SCH 527123 on neutrophil migration to IL-8, we saw no differences between day 1 pre-dosing and after 8 days of treatment with SCH 527123, nor difference on the response compared to placebo (Fig. 4).

4. Discussion

This study demonstrated that treatment of mild allergic

asthmatics with the dual CXCR1/CXCR2 antagonist SCH 527123 significantly reduced the number of neutrophils within the peripheral blood and sputum. As we aimed to assess the effects of SCH 527123 on neutrophil levels and function in blood, bone marrow and sputum of asthmatic donors using a crossover study design, subjects with stable, mild asthma were most suitable for this study. Patients with severe asthma were studied in a separate clinical trial [22]. There is increased recognition of the neutrophilic asthma phenotype [24] with IL-8 being the primary cytokine within the airways [17,25]. The chemokine receptor CXCR2 plays an integral role in the accumulation of neutrophils [26] and CXC chemokines including IL-8, and their receptors CXCR1 and CXCR2, have been shown to be up-regulated in the airways during an asthma exacerbation [27]. Inhibition of neutrophil chemotaxis by blocking CXCR1 and CXCR2 may offer an alternative therapeutic strategy for asthmatic patients with predominately neutrophilic inflammatory profiles. This is of particular clinical importance as these patients often respond poorly to corticosteroids [28]. By targeting cells, which rely on CXCR1 and CXCR2-mediated chemotaxis for accumulation to airway tissue, SCH 527123 poses as a novel therapeutic agent to exert selective effects on inflammation.

Together with the *in vitro* data showing that pre-incubation with SCH 527123 inhibited migration of neutrophils purified from these same subjects, we propose that the mechanism of action of SCH 527123 *in vivo* is mediated through blocking the egress of neutrophils from the bone marrow, and this is consistent with previous reports [29]. Any corresponding increase in the number of neutrophils in the bone marrow may be difficult to observe in this small study due to the relatively larger number of neutrophils in bone marrow compared to peripheral blood.

Our *in vitro* experiments demonstrate that the chemotactic response of neutrophils purified from peripheral blood is susceptible to CXCR1/CXCR2 blockade, thus oral dosing with SCH 527123 could indeed suppress trafficking of neutrophils from the periphery to the airways. The chemotactic response to IL-8 but not fMLP was blocked by SCH 527123, confirming the drug effects are specifically mediated through CXCR1 and CXCR2. Our findings of reduced airway neutrophilia following treatment with SCH 527123 are consistent with previous reports [21,22]. In a study conducted in patients with severe asthma, SCH 527123 caused a mean reduction of 36.3% in sputum neutrophil percentage [22] versus a 64% reduction observed in the current study. In the current study, we have not addressed the consequence of reducing neutrophil levels in the bone marrow, circulation or airways.

We also demonstrated that a subset of mature bone marrow-derived neutrophils studied *in vitro* responded to IL-8 and SCH 527123, supporting the notion that oral doses of SCH 527123 could suppress trafficking of neutrophils from bone marrow to the periphery. The reasons for weaker effects of IL-8 and SCH 527123 in bone marrow neutrophils may reflect differing expression and roles of chemokine receptors during cell maturation. We do not believe the reduction in neutrophils within the periphery reflects an effect of SCH 527123 on granulopoiesis, as our data show no significant change in the number of immature neutrophils or the overall neutrophil count in the bone marrow. In addition, we found no treatment effects on the bone marrow myeloid to erythroid ratio, erythroid cells, or any other cell type seen in the study (data not shown). These findings are consistent with a report showing that treatment with a selective CXCR2 antagonist, AZD5069, did not adversely affect the mobilization of human neutrophils from the bone marrow into the peripheral circulation. Importantly, these authors also reported no effect on neutrophil phagocytosis or the oxidative burst response to bacterial pathogens [30], and these data are consistent with the safety profile we and others have reported during treatment with SCH 527123.

The study design permitted comparison of neutrophil function following treatment with SCH 527123 and placebo. When neutrophils were purified from the peripheral blood and bone marrow of mild asthmatic subjects post-dosing and the chemotactic response to IL-8 was measured *ex vivo*, we did not observe a difference between the two treatment groups. There are several limitations to the interpretation of the *ex vivo* experiments. The neutrophils from bone marrow were relatively insensitive to direct blocking by SCH 527123, thus the conclusions from the *ex vivo* experiments in bone marrow are limited. Also, with SCH 527123 being an allosteric antagonist of both CXCR1 and CXCR2 [26], we expected neutrophil CXCR1 and CXCR2 receptors to be occupied by drug post-dosing, rendering the cells unresponsive to the *ex vivo* chemotactic effects of IL-8. During the purification of neutrophils, repeated washing of cells may have removed SCH 527123 from receptors. It has been shown that dissociation of SCH 527123 from CXCR1 is more rapid than from CXCR2, thus *ex vivo* effects would be more optimally tested using a chemokine that acts exclusively through CXCR2 [26]. The functional experiments reported here were all conducted using IL-8, which is known to induce chemotaxis of neutrophils through both CXCR1 and CXCR2 [31,32]. Although we performed identical experiments using Gro- α (which acts only through CXCR2), the results could not be interpreted due to lack of chemotactic effect using our experimental approach, and thus have not been reported here. We cannot eliminate the possibility that IL-8-induced migration observed during *ex vivo* experiments was mediated through CXCR1 receptors unoccupied by the drug.

CXCR2 is expressed on leukocytes and structural cells that play central roles in asthma [33,34], and under certain conditions eosinophils are responsive to CXC chemokines. Eosinophil expression of CXCR2 is closely related to asthma exacerbations [28], and eosinophil migrational responses to IL-8 are restricted to individuals with peripheral blood eosinophilia [34]. Thus, we also examined whether eosinophil levels in mild asthmatic subjects could be reduced by SCH 527123 treatment. Following dosing, we observed no effect of SCH 527123 on eosinophils in the peripheral blood or induced sputum, which is perhaps not surprising given the low eosinophil levels in these subjects, and intact signaling of more potent eosinophil chemoattractants such as eotaxin.

SCH 527123 was developed to target CXCR2 in order to inhibit migration of neutrophils from bone marrow into blood, and thus reduced levels of neutrophils in the circulation are an expected outcome. Clinical trials of SCH 527123 have selected a blood neutropenia of $1.5 \times 10^6/\text{mL}$ as a pre-specified cause for treatment discontinuation. In the current study, 4 of 19 subjects developed neutropenia, and similarly in a group of asthmatic patients also treated with 30 mg SCH 527123 for 4 weeks, 2 of 22 were discontinued for the same reason [22]. Longer-term treatment with SCH 527123 in COPD patients has also been associated with discontinuation of treatment due to adverse events, driven by dose-related decrease in the absolute neutrophil count below $1.5 \times 10^6/\text{mL}$ [35]. Other adverse effects did not differ between SCH 527123 and placebo in these studies. Discontinuation due to neutropenia is not unique to SCH 527123, as four of 58 patients with moderate-to-severe COPD were discontinued from treatment with another CXCR2 antagonist due to a drop of neutrophil levels below the pre-specified level of $1.0 \times 10^6/\text{mL}$ [36].

This study demonstrated that blockade of CXCR1 and CXCR2 reduces the level of neutrophils in the airways of asthmatic subjects, mediated in part by a reduction in neutrophil migration from the blood. We provide evidence that oral administration of 30 mg SCH 527123 for 8 days is effective in reducing trafficking of neutrophils to the blood and airways without having a detrimental effect on bone marrow cellularity.

Disclosure

JS was previously employed by Merck & Co., Inc., L-PB, PMO and GMG have received research support from Schering Plough. PMO and GMG are on the Advisory Board for Merck Frost. CT, BMS, DM, RMW, KJH, and JM have declared that they have no conflict of interest.

Clinical implications

These offer mechanistic insight into how a novel therapeutic approach of CXCR2 antagonism may regulate neutrophilic inflammation in asthmatic subjects.

Acknowledgements

This study was funded by Schering Plough with administrative support provided by AllerGen NCE.

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