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The effect of phosphatidylinositol-3 kinase inhibition on matrix metalloproteinase-9 and reactive oxygen species release from chronic obstructive pulmonary disease neutrophils

V. Gupta [a,*], A. Khan [a], A. Higham [a], J. Lemon [a], S. Sriskantharajah [b], A. Amour [b], E.M. Hessel [b], T. Southworth [a], D. Singh [a]

[a] University of Manchester, Medicines Evaluation Unit, Centre for Respiratory Medicine and Allergy, Institute of Inflammation and Repair, Manchester Academic Health Science Centre, University Hospital of South Manchester, NHS Foundation Trust, Manchester M23 9LT, UK
[b] Refractory Respiratory Inflammation Discovery Performance Unit, GlaxoSmithKline, Stevenage, UK

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ABSTRACT

Background: Chronic Obstructive Pulmonary Disease (COPD) is characterised by increased neutrophilic inflammation. A potential novel anti-inflammatory target in COPD is phosphatidylinositol-3 kinase (PI3 kinase), which targets neutrophil function. This study evaluated the effects of selective PI3Kδ inhibition on COPD blood and sputum neutrophils both in the stable state and during exacerbations.

Methods: Blood and sputum neutrophils from stable and exacerbating COPD patients were cultured with the corticosteroid dexamethasone, a pan PI3 kinase inhibitor (ZSTK474), a δ selective PI3 kinase inhibitor (GSK045) and a p38 mitogen activated protein (MAP) kinase inhibitor (BIRB 796); matrix metalloproteinase (MMP)-9 and reactive oxygen species (ROS) release were analysed.

Results: PI3Kδ inhibition significantly reduced MMP-9, intracellular ROS and extracellular ROS release from blood neutrophils (45.6%, 30.1% and 47.4% respectively; $p < 0.05$) and intracellular ROS release from sputum neutrophils (16.6%; $p < 0.05$) in stable patients. PI3Kδ selective inhibition significantly reduced stimulated MMP-9 (36.4%; $p < 0.05$) and unstimulated and stimulated ROS release (12.6 and 26.7%; $p < 0.05$) from blood neutrophils from exacerbating patients. The effects of the p38 MAP kinase inhibitor and dexamethasone in these experiments were generally lower than PI3Kδ inhibition.

Conclusion: PI3Kδ selective inhibition is a potential strategy for targeting glucocorticoid insensitive MMP-9 and ROS secretion from COPD neutrophils, both in the stable state and during exacerbations.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterised by an excessive and persistent immune response to the inhalation of noxious particles [1]. There are increased neutrophil numbers in the lungs of COPD patients [2], with a further increase during exacerbations [3]. There is evidence of increased activity of COPD neutrophils, with enhanced secretion of pro-inflammatory mediators involved in the pathophysiology of COPD, such as proteases [4] and reactive oxygen species (ROS) [5].

Corticosteroids are the most widely used anti-inflammatory therapy for COPD patients, but these drugs have limited clinical benefits [6]. Lung neutrophils show reduced expression of the glucocorticoid receptor [7], thus limiting the effects of corticosteroids on neutrophilic lung inflammation. There is a need for novel therapies to treat neutrophilic inflammation in COPD.

Phosphatidylinositol-3 kinases (PI3 kinases) are a potential therapeutic target for the treatment of COPD. These intracellular enzymes are involved in cell metabolism, growth and repair [8]. Class I PI3 kinases catalyse the formation of PIP_3; this lipid second messenger controls cell metabolism and activation pathways through phosphorylation of Akt and other proteins [9]. Class 1 PI3 kinases are composed of a regulatory subunit and a catalytic subunit; either p110α, p110β, p110γ or p110δ. The γ and δ isoforms are predominantly leucocyte specific, and so there is currently much interest in developing compounds that selectively inhibit these isoforms for the treatment of inflammatory diseases.

Studies using peripheral blood neutrophils from healthy subjects have shown that both δ and γ isoforms are involved in ROS release [10], and that pan PI3K inhibition suppresses cytokine [11] and matrix-metalloproteinase 9 (MMP-9) [12] secretion. In addition, PIP_3 levels and PI3Kδ gene expression are increased in COPD blood neutrophils compared to healthy controls [13]. This suggests an important role for PI3Kδ in the regulation of neutrophil activity in COPD patients. However, the effects of PI3 kinase δ selective inhibition on the secretion

* Corresponding author.
E-mail address: vgupta@meu.org.uk (V. Gupta).

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of inflammatory mediators from COPD neutrophils has not been investigated.

The aim of this study was to understand the anti-inflammatory effects of selective PI3Kδ inhibition on COPD neutrophils. Importantly, neutrophils from the blood and sputum were studied, with investigations performed in the stable clinical state and during exacerbations. We focused on ROS and MMP-9 release from neutrophils, as these are relevant to the pathophysiology of COPD [14].

2. Methods

2.1. Study subjects

Patients were recruited with a previous diagnosis of COPD, with an FEV1/FVC ratio < 70%, age > 40 and at least a 10 pack year history of smoking. Patients were excluded if there was a history of active malignancy, asthma or any other inflammatory disease. Patient demographics are summarised in Table 1. Stable patients were recruited only if they were free from respiratory infections in the preceding 6 weeks. All patients gave written informed consent. Approval was obtained from the local ethics committees GM South (05/Q1402/41), NW-Preston (10/H1016/25) and GM East (10/H1003/108).

2.2. Exacerbations

COPD patients presenting with exacerbations were recruited from outpatient clinics and respiratory wards at the University Hospital South Manchester, UK. Exacerbations were defined as increased respiratory symptoms for 2 days with at least 1 major symptom (dyspnoea, sputum purulence and sputum volume), and another major or a minor symptom (wheeze, cold, sore throat and cough) [15]. Exacerbating patients seen in the clinic had been asked to keep diary cards of their symptoms and were only included if they had not received antibiotics or steroids for 6 weeks prior to their clinic visit. Patients recruited from wards were sampled within 24 h of admission and receiving treatment. Patients were sampled a mean of 2.9 ± 0.5 (s.d.) days after symptoms started.

2.3. Spirometry and sputum processing

Spirometry was carried out according to American Thoracic Society (ATS) guidelines. All stable patients had sputum induced; spontaneous or induced sputum was sampled in patients with exacerbations. Sputum was induced and processed with dithiothreitol (DTT) using established protocols [16], and cells (2×10^6/ml) resuspended in phosphate buffered saline (Sigma Aldrich, Poole, UK). Cytospin preparations were made (Cytospin 4, Shandon, Runcorn, UK) and stained with Rapi-diff (Triangle, Skelmersdale, UK). Non-squamous cells (400) were counted and differential cell counts were obtained as percentage of total non-squamous cells. Cell viability was analysed by trypan blue exclusion.

2.4. Blood neutrophil isolation

Venous blood (5 ml) was layered over Mono-poly resolving medium (3 ml) (MP Biomedicals, Cambridge, UK) and centrifuged (800 *g* for 45 min at 18 °C). Blood neutrophil isolation is described in detail in the online supplement. The resulting cell suspension was ≥97% neutrophils.

2.5. Cell culture: MMP-9 release

Isolated neutrophils were pre-treated (1 h) with a pan PI3K inhibitor; ZSTK474 (Selleck chemicals, Suffolk, UK), a selective PI3K δ inhibitor; GSK045 (GlaxoSmithKline), (Patent W02009147187A1; manuscript in preparation), the p38 inhibitor BIRB 796 (Selleck chemicals), and dexamethasone (Sigma-Aldrich); the compounds were all used at 1–1000 nM in stable blood neutrophils. However, for blood neutrophils obtained from exacerbating patients only 1000 nM was used as it was felt to be unsafe to venesect large volumes of blood from unwell patients. pIC50s for the PI3 kinase inhibitors are shown in the online supplement. Thereafter, cells were stimulated (30 min) with fMLP (10 nM) (Sigma-Aldrich); this concentration was chosen as it was the sup-optimal concentration for MMP-9 release (see Supplementary Fig. 1). Cell-free supernatants were collected and stored (−80 °C) for MMP-9 analysis.

MMP-9 was measured by ELISA according to the manufacturer's instructions (R&D systems, Abingdon, UK; limits of detection 31.2–2000 pg/ml).

Table 1
A summary of patient demographics. All values are expressed as mean ± s.d., apart from *, expressed as median (range); * indicates data only available for $n = 6$. IC: intracellular, EC: extracellular, FEV1: forced expiratory volume in 1 s post 200 μg inhaled salbutamol, FVC: Forced Vital Capacity, ICS: inhaled corticosteroids, LAMA: long acting muscarinic antagonist, LABA: long acting beta agonist, BDP: beclomethasone diproprionate equivalent, MMRC: Modified Medical Research Council Questionnaire.

	Stable COPD				Exacerbations	
	Blood MMP-9 ($n = 11$)	Blood IC ROS ($n = 6$)	Blood EC ROS ($n = 8$)	Sputum IC ROS ($n = 11$)	Blood MMP-9 ($n = 15$)	Blood IC ROS ($n = 12$)
Age (years)	67 (54–74)*	65 (56–69)*	69 (55–75)*	71 (58–75)*	66 (60–84)*	66 (60–81)*
Sex (M/F)	7/4	4/2	5/3	11/0	11/4	9/3
FEV1 (litres)	1.77 ± 0.63	1.90 ± 1.45	1.88 ± 0.70	1.85 ± 0.72	1.13 ± 0.38	1.14 ± 0.40
FEV1 % pred	66.12 ± 13.68	58.86 ± 34.15	71.33 ± 22.17	57.47 ± 16.98	44.67 ± 11.44	44.08 ± 12.08
FEV1/FVC ratio	51.37 ± 10	45.47 ± 16.28	43.27 ± 22.42	47.21 ± 12.07	36.27 ± 5.87	36.75 ± 6.50
Pack years	40.13 (20–67)*	44 (31.8–101)*	42.5 (25.3–138.1)*	41.8 (20–82.8)*	40.0 (17–72)*	39.1 (17–72)*
Smoking (Ex/C)	6/5	3/3	5/3	6/5	9/6	8/4
ICS (Y)	6	6	6	6	11	11
ICS dose (BDP)	1400 (250–2000)*	2000 (0−2000)*	2000 (1000−2000)*	1000 (0–2000)*	2000 (0–2000)*	2000 (0–2000)*
LAMA (Y)	4	5	7	6	10	9
LABA (Y)	7	6	6	8	15	12
MMRC score	1.55 ± 0.34	3 ± 1.0	1.83 ± 0.75*	1.27 ± 1.0		