A Randomized, Placebo-controlled Trial of Roflumilast

Effect on Proline-Glycine-Proline and Neutrophilic Inflammation in Chronic Obstructive Pulmonary Disease

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Abstract

Rationale: Roflumilast is a therapeutic agent in the treatment of chronic obstructive pulmonary disease (COPD). It has antiinflammatory effects; however, it is not known whether it can affect a biologic pathway implicated in COPD pathogenesis and progression. The self-propagating acetyl-proline-glycine-proline (AcPGP) pathway is a novel means of neutrophilic inflammation that is pathologic in the development of COPD. AcPGP is produced by extracellular matrix collagen breakdown with prolyl endopeptidase and leukotriene A_4 hydrolase serving as the enzymes responsible for its production and degradation, respectively.

Objectives: We hypothesized that roflumilast would decrease AcPGP, halting the feed-forward cycle of inflammation.

Methods: We conducted a single-center, placebo-controlled, randomized study investigating 12 weeks of roflumilast treatment added to current therapy in moderate-to-severe COPD with chronic bronchitis. Subjects underwent sputum and blood analyses, pulmonary function testing, exercise tolerance, and quality-of-life assessment at 0, 4, and 12 weeks.

Measurements and Main Results: Twenty-seven patients were enrolled in the intention-to-treat analysis. Roflumilast treatment decreased sputum AcPGP by more than 50% ($P < 0.01$) and prolyl endopeptidase by 46% ($P = 0.02$), without significant improvement in leukotriene A_4 hydrolase activity compared with placebo. Roflumilast also reduces other inflammatory markers. There were no significant changes in lung function, quality of life, or exercise tolerance between roflumilast- and placebo-treated groups.

Conclusions: Roflumilast reduces pulmonary inflammation through decreasing prolyl endopeptidase activity and AcPGP. As expected for lower AcPGP levels, markers of neutrophilic inflammation are blunted. Inhibiting this self-propagating pathway lessens the overall inflammatory burden, which may alter the natural history of COPD, including the risk of exacerbation.

Clinical trial registered with www.clinicaltrials.gov (NCT 01572948).

Keywords: COPD; roflumilast; neutrophil; proline-glycine-proline; prolyl endopeptidase

Chronic obstructive pulmonary disease (COPD) is currently the third leading cause of death and the sixth leading cause of disability in the United States (1). Chronic neutrophilic inflammation is a hallmark of the disease and directly impacts morbidity and mortality (2–4). At present, treatments are aimed primarily at the control of dyspnea and the prevention of complications, such as acute exacerbations (AECOPD), which drive most COPD-related morbidity and costs (5, 6). Inhaled

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At a Glance Commentary

Scientific Knowledge on the Subject: Roflumilast is a therapeutic used in chronic obstructive pulmonary disease (COPD) in part because of its antiinflammatory effects. However, it is not known whether it can affect a biologic pathway implicated in COPD pathogenesis and progression. The self-propagating acetyl-proline-glycine-proline pathway is a novel means of neutrophilic inflammation that is pathologic in the development of COPD. Whether roflumilast treatment is effective in blunting this pathway is unknown.

What This Study Adds to the Field: Roflumilast reduces pulmonary inflammation through decreasing prolyl endopeptidase activity and acetyl-proline-glycine-proline. Inhibiting this self-propagating pathway lessens the overall inflammatory burden, which may alter the natural history of COPD, including the risk of exacerbation.

therapies, including bronchodilators and inhaled corticosteroids (ICS), are the mainstay of this management strategy but are only modestly effective even when used in combination. No intervention other than smoking cessation and the use of supplemental oxygen in those patients with resting hypoxemia has been definitively shown to alter the natural history of the disease (7, 8). Major limitations to disease modification include the absence of any drug that attenuates the ongoing airway inflammation that persists even after smoking cessation (9, 10). Roflumilast is a selective phosphodiesterase-4 inhibitor, and the first medication approved exclusively for decreasing AECOPD, in part because of its role in reducing pulmonary inflammation and neutrophil burden (11–13).

Roflumilast increases intracellular cAMP in inflammatory cells, bronchial epithelia, and bronchial smooth muscle, and reduces leukotriene B_4 (LTB_4), reactive oxygen species, and tumor necrosis factor-α *in vitro* (14, 15). These antiinflammatory effects also occur in the short- and long-term where roflumilast has also been demonstrated to reduce sputum neutrophil count, eosinophil count, soluble IL-8, and neutrophil elastase, and lead to improvement in lung function and reduction in AECOPD risk (12).

However, neutrophil elastase, IL-8, and neutrophil burden are end products of several inflammatory pathways and are not specific for roflumilast's mechanistic target. In contrast, proline-glycine-proline (PGP) and its acetylated form (AcPGP) are tripeptide collagen fragments generated from extracellular matrix breakdown that act as potent neutrophil chemoattractants, and are detected in sputum of patients with COPD (16–19). AcPGP and PGP are generated through a multistep proteolytic pathway with prolyl endopeptidase serving as the critical rate-limiting enzyme (19). Leukotriene A_4 hydrolase (LTA_4H) is the enzyme responsible for degrading PGP and terminating PGP-mediated neutrophilic influx in normal conditions (20). LTA_4H is classically known as an epoxide hydrolase that converts LTA_4 to LTB_4 (21) and also has aminopeptidase activity specific for degrading PGP (20). Cigarette smoke selectively inactivates LTA_4H's aminopeptidase activity without affecting the hydrolase. This novel pathway of neutrophilic inflammation, unlike the "classic" mode associated with IL-8, has the potential to become self-propagating (17). In addition to its role in pathogenesis, PGP levels may also serve as a marker of AECOPD risk (22).

We hypothesize the antiinflammatory activity of roflumilast is caused by direct and indirect effects on neutrophils by interfering with the AcPGP pathway. First, roflumilast may impair AcPGP production by decreasing prolyl endopeptidase, the oligopeptidase responsible for its release from extracellular matrix collagen fragments (19, 23). Second, it may also stimulate PGP degradation by direct interaction with the enzyme LTA_4H and increasing its aminopeptidase function. Either one or both of these mechanisms would explain roflumilast's capacity to blunt chronic neutrophilic inflammation in patients with moderate to severe COPD and reduce overall disease burden. We also posit that these meaningful reductions in biomarkers of pulmonary inflammation and sputum neutrophilia may trend with improvement in pulmonary function, sputum scores, and quality of life in stable moderate-to-severe COPD.

Methods

Patient Selection

Subjects were recruited from a single center to participate in a 12-week double-blinded, placebo-controlled, randomized control trial. The Institutional Review Board at the University of Alabama at Birmingham approved the conduct of the study (protocol number #F111129001) and the study is listed in ClinicalTrials.gov (NCT01572948). The study was granted an investigational new drug exemption by the Food and Drug Administration. Patients underwent informed consent and were included if they were greater than 40 years old, had a diagnosis of moderate-to-severe COPD as defined by Global Initiative for Chronic Obstructive Lung Disease criteria (24), were current or former cigarette smokers with more than 10 pack-years of total consumption, and had chronic bronchitis defined by chronic cough and sputum production lasting at least 3 months for 2 consecutive years,. Exclusion criteria include a diagnosis of asthma as defined by the American Thoracic Society/European Respiratory Society guidelines, clinically significant bronchiectasis, known sensitivity to roflumilast, the use of other methylxanthines (specifically theophylline) within 1 month of screening, changes to maintenance COPD therapy within 1 month of screening, and other factors as listed in the online supplement.

Study Protocol

The study was divided into six visits as outlined in Figure E1 in the online supplement. Visit 1 (Week −1) activities included informed consent, demographic and medical history evaluation, pulmonary function testing including prebronchodilator and post-bronchodilator FEV_1 and FVC, and blood and induced sputum collection. Visit 2 (Week 0) included interval history; completion of St. George's Respiratory Questionnaire (SGRQ) and Breath, Cough, and Sputum Scale (BCSS) questionnaires; 6-minute-walk distance (6MWD); and randomization by sealed envelope. Subjects were randomized to identical white tablets containing a 30-day supply of either roflumilast, 500 μg or placebo by a block randomization schema using a block size of four and an allocation ratio of 1:1. Block randomization was stratified by current smoking status and ICS use. This was done based on known

associations between cigarette smoke use and increased PGP/AcPGP levels (17, 25) and uncertainty surrounding roflumilast use in the setting of ICS (11, 13, 26). The latter issue has been clarified somewhat by the recent publication of the REACT study demonstrating that as compared with placebo, roflumilast reduced the risk of exacerbations, including those requiring hospitalization, when used in conjunction with ICS in patients with chronic bronchitis (27).

Visit 3 (Week 4) included medical history review, spirometry, and blood and induced sputum collection. Visit 4 (Week 8) events included medical history update and spirometry. Visit 5 (Week 12) included medical history review, pulmonary function testing, quality-of-life questionnaires, blood and induced sputum collection, and 6MWD. Visit 6 (Week 14) was telephone follow-up after study completion to ensure no symptoms developed following investigational product cessation. Compliance was assessed by counting the number of pills participants returned at each follow-up visit.

Pulmonary Function and Exercise Tolerance Testing

Prebronchodilator and post-bronchodilator spirometry was performed on all subjects according to American Thoracic Society/European Respiratory Society standards (28) and 6MWD was performed according to American Thoracic Society standards (29).

Induced Sputum

Induced sputum using inhaled 3% hypertonic nebulized saline followed a well-established protocol by a trained respiratory therapist (12, 30, 31) and was used for all collections. Induced sputum was diluted in phosphate-buffered saline (1:4).

Quality-of-Life Determination

The SGRQ is a 50-item quality-of-life questionnaire with a minimal clinically important difference of a change in score of 4 points (32). The BCSS is a validated questionnaire examining the impact of cough- and sputum-related symptomatology with a substantial difference of a change of greater than 1.0 (33).

Plasma and Sputum Preparation

Phree columns (Phenomenex, Torrance, CA) were loaded with 1 ml of 60:40 methanol/acetonitrile. $^{13}C^{15}N$-labeled AcPGP/$^{13}C^{15}N$-labeled PGP mix was added (final concentration of 10 ng/ml) directly onto the chromatography column with 200 μl of patient plasma or sputum to measure recovery. The recovery (mean ± SD) was 38.4 ± 10.3% for AcPGP and 35.7 ± 23.1% for PGP after filtration. The columns were covered and centrifuged at 1,500 × *g* for 60 minutes followed by an additional methanol/acetonitrile wash. The solution that had passed through the column was evaporated to dryness using a REACTI-VAP III system (Fisher Scientific, Pittsburgh, PA). The residue was dissolved in 100 μl of phosphate-buffered saline and used for liquid chromatography electrospray ionization tandem mass spectroscopy.

Biomarker Analyses

Sputum cell count was performed using a hemocytometer at ×40 magnification. AcPGP, PGP, and LTA_4H aminopeptidase activity were detected by liquid chromatography electrospray ionization tandem mass spectroscopy as previously described (16, 17, 20, 25). LTA_4H (Uscn, Wuhan, China), LTB_4 (R&D Systems, Minneapolis, MN), IL-8 (CXCL8/IL-8 Quantikine kit; R&D Systems), and myeloperoxidase (MPO) (CalBiochem) were detected in induced sputum by commercially available enzyme immunoassays (17, 34, 35). Neutrophil elastase was detected by a commercially available chemiluminescence kit (CalBiochem, Billerica, MA). Prolyl endopeptidase activity was measured via immunofluorescence assay as previously described (19, 23, 36).

Statistical Analyses

The primary outcome for the study was a change in induced sputum AcPGP at 12 weeks post-randomization in an intention-to-treat analysis. The study was powered to detect a 50% reduction in sputum AcPGP based on reductions observed in the COPD Clinical Research Network Macrolide trial (22). To achieve these results with a power of 0.80 and an alpha of 0.05, 24 patients (n = 12 per group) were needed. Additional analyses were performed to compare the between-group changes in plasma AcPGP, sputum neutrophil counts, additional sputum biomarkers, the BCSS and SGRQ scores, and changes in post-bronchodilator FEV_1 at the 12-week visit. All secondary analyses included all patients who were enrolled (intention-to-treat). Normal distribution was confirmed with the Shapiro-Wilk test. Bivariate analyses were conducted with the use of a two-tailed Fisher exact test for categorical data and two-tailed Student *t* test or Mann-Whitney *U* test for continuous data as appropriate. Repeated measures analysis of variance or Friedman test was used to determine changes in sputum and plasma biomarker values at the three time points (Weeks 0, 4, and 12) and paired Student *t* test or Wilcoxon matched-pair signed rank test was used to determine changes in sputum and plasma biomarker values at Weeks 0 and 12 for normally or nonnormally distributed values, respectively. All analyses were performed with SPSS Software (Version 22.0; IBM Corporation, Armonk, NY) and *P* values less than 0.05 were considered statistically significant. Figures were designed in Prism Version 5 (GraphPad Software Inc., La Jolla, CA).

Results

Patients

From 2012 to 2014, a total of 42 subjects were screened, of which 27 were enrolled. All patients received their study drug, and one participant in each study arm was lost to follow-up (Figure 1). Baseline characteristics were not statistically different between the two groups (Table 1). Subjects were 62 ± 7 years old, 63% male, 70% non-Hispanic white, with post-bronchodilator FEV_1 of 44 ± 14% predicted. In total, 59% were current smokers with a 45 ± 22 pack-year history, 26% used supplemental oxygen, and 59% used either ICS alone or in combination with long-acting β-agonist therapy. Baseline quality-of-life scores (SGRQ), sputum scores (BCSS), and exercise capacity (6MWD) were comparable between placebo- and roflumilast-treated subjects. No participants developed AECOPD during the course of the study.

Primary Efficacy Variables

Sputum AcPGP and PGP. Baseline sputum AcPGP (0.64 ± 0.21 vs. 0.74 ± 0.25 ng/ml; $P = 0.76$) and PGP (0.42 ± 0.14 vs. 0.59 ± 0.19 ng/ml; $P = 0.45$) were not statistically different among subjects treated with roflumilast or placebo, respectively. As seen in Figure 2A, roflumilast treatment reduced sputum AcPGP at 12 weeks of therapy

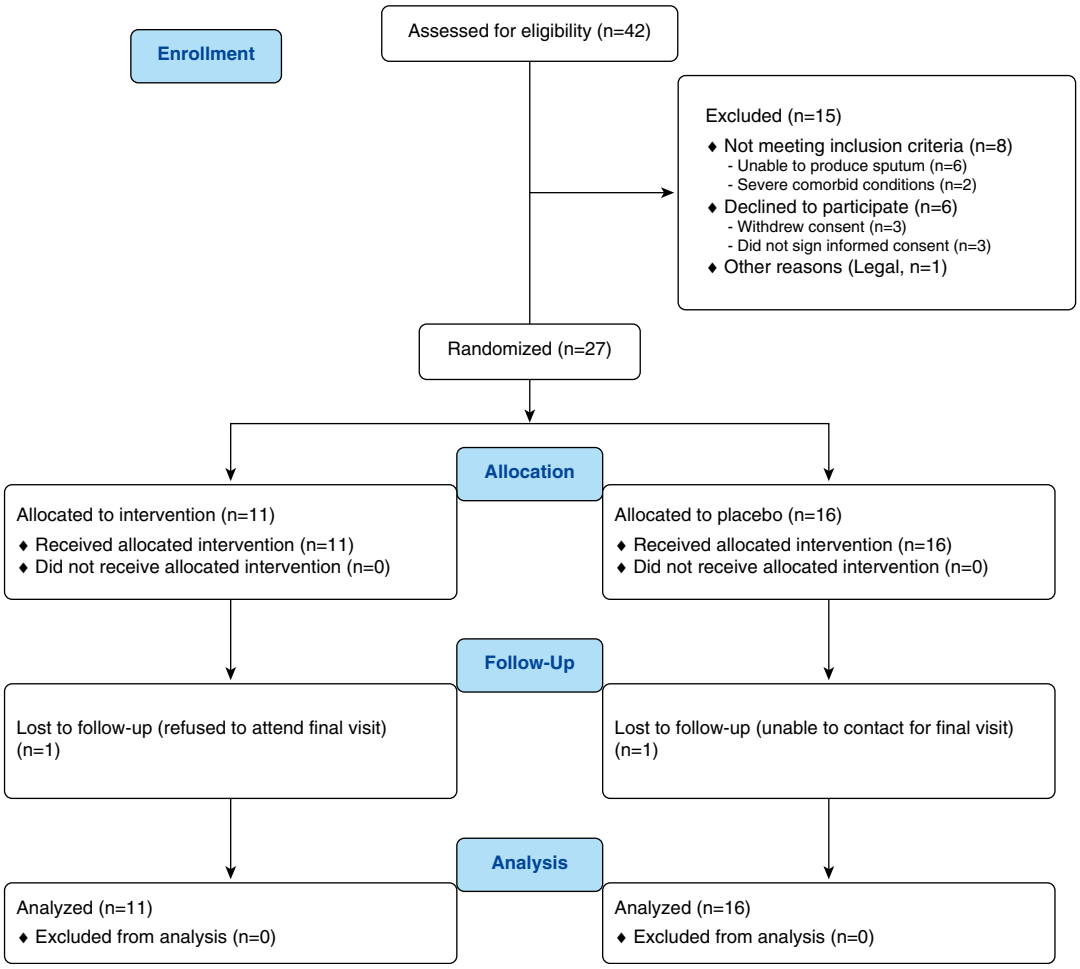

Figure 1. Consolidated Standards of Reporting Trials flow diagram.

(0.30 ± 0.11 compared with 0.70 ± 0.18 ng/ml in placebo; $P = 0.04$), corresponding to a greater than 50% reduction in sputum AcPGP ($P = 0.01$ by Wilcoxon matched-pair signed rank test) (Figure 2B). In a separate, per-protocol analysis, sputum AcPGP was decreased after 12 weeks of roflumilast treatment (0.27 ± 0.11 compared with 0.81 ± 0.21 ng/ml in placebo; $P = 0.03$). Although sputum PGP levels were decreased after 12 weeks of roflumilast therapy, these were not statistically different from findings in the placebo group (Figure 2C) (0.29 ± 0.08 compared with 0.52 ± 0.28 ng/ml in placebo; $P = 0.44$).

Secondary Efficacy Variables

Effects on AcPGP/PGP generation in sputum. Baseline sputum prolyl endopeptidase was not statistically different between roflumilast- and placebo-treated subjects (0.81 ± 0.62 vs. 0.65 ± 0.34 nM, respectively; $P = 0.25$). As seen in Figure 3A, 12 weeks of roflumilast treatment significantly reduces prolyl endopeptidase compared with placebo (0.44 ± 0.15 vs. 0.73 ± 0.42 nM; $P = 0.02$). Sputum prolyl endopeptidase activity had a modest correlation with sputum AcPGP ($r = 0.39$; $P = 0.059$).

Effects on PGP degradation in sputum. Baseline LTA_4H (14.4 ± 3.5 vs. 14.1 ± 3.4 ng/ml; $P = 0.96$) and LTA_4H aminopeptidase activity (2.7 ± 1.0 vs. 7.4 ± 1.8 ng/ml/min per ng/enzyme; $P = 0.09$) were comparable between roflumilast- and placebo-treated subjects, respectively. As seen in Figure 3B, roflumilast treatment results in a reduction of sputum LTA_4H (3.2 ± 0.6 vs. 9.6 ± 2.3 ng/ml in placebo; $P = 0.04$). Although there was a 4.5-fold improvement in aminopeptidase activity in the roflumilast-treated group (Figure 3C; $P = 0.08$), there were no statistical differences in aminopeptidase activity between roflumilast- and placebo-treated subjects (13.3 ± 5.0 vs. 7.6 ± 3.5 ng/ml/min per ng/enzyme; $P = 0.36$).

Plasma PGP/AcPGP markers. Although the presence and importance of sputum PGP/AcPGP have been described, little is known about its presence in the systemic circulation. We measured plasma PGP and AcPGP according to roflumilast intervention compared with placebo. Plasma AcPGP (0.28 ± 0.05 vs. 0.26 ± 0.02 ng/ml; $P = 0.69$) and PGP (0.10 ± 0.03 vs. 0.12 ± 0.02 ng/ml; $P = 0.51$) were comparable at baseline between placebo and roflumilast groups. As seen in Figure 4, roflumilast treatment did not result in reductions of either PGP or AcPGP in plasma.

Sputum neutrophil cell count and inflammatory markers. Sputum MPO (110 ± 29 vs. 108 ± 21 ng/ml; $P = 0.94$), LTB_4 (658 ± 214 vs. 675 ± 158 pg/ml; $P = 0.94$), IL-8 (2,811 ± 460 vs. 2,950 ± 385 pg/ml; $P = 0.82$), and neutrophil elastase

Table 1. Baseline Characteristics

	Placebo (*n* = 16)	Roflumilast (*n* = 11)
Age, yr	61 ± 8	62 ± 7
White race	12 (75%)	7 (64%)
Male sex	10 (63%)	7 (64%)
Current smoker	10 (63%)	6 (55%)
Lifetime pack-year history	44 ± 19	47 ± 26
GOLD stage, median (range)	3 (2–4)	3 (2–4)
Supplemental oxygen use	4 (25%)	3 (27%)
Supplemental oxygen amount, L/min	2 ± 0	1.7 ± 0.6
LAMA use	8 (50%)	6 (55%)
ICS or LABA/ICS use	10 (63%)	6 (55%)
Post-bronchodilator FEV_1, % predicted	44 ± 16	45 ± 12
Post-bronchodilator FVC, % predicted	69 ± 20	72 ± 17
FEV_1/FVC ratio	0.48 ± 0.12	0.53 ± 0.12
SGRQ total	49 ± 13	56 ± 13
BCSS	4.6 ± 2.4	4.6 ± 2.7
6-min-walk distance, ft	972 ± 255	739 ± 203

Definition of abbreviations: BCSS = Breathlessness Cough and Sputum Scale; GOLD = Global Initiative for Chronic Obstructive Lung Disease; ICS = inhaled corticosteroid; LABA = long-acting β-agonist; LAMA = long-acting muscarinic antagonist; SGRQ = St. George's Respiratory Questionnaire.
All results are mean ± SD or number of patients (%).
Differences between groups for each category listed were not statistically different ($P > 0.05$).

(6.9 ± 3.1 vs. 7.1 ± 2.7 ng/ml; $P = 0.96$) were similar at baseline between roflumilast and placebo groups, respectively. As seen in Figure 5, roflumilast treatment at 12 weeks results in significant reduction of MPO (39 ± 9 vs. 133 ± 23 ng/ml; $P = 0.003$), LTB_4 (176 ± 77 vs. 903 ± 248 pg/ml; $P = 0.03$), and neutrophil elastase (1.6 ± 0.8 vs. 8.7 ± 2.8 ng/ml; $P = 0.03$), with a trend toward lower IL-8 (1,530 ± 512 vs. 2,680 ± 348 pg/ml; $P = 0.08$) levels in sputum. The concomitant reductions in LTA_4H and LTB_4 with relative sparing of aminopeptidase activity reinforce prior observations of selective LTA_4H inhibition. Although there was a reduction in overall sputum neutrophil burden after 12 weeks of therapy, it did not reach statistical significance (Figure 5E) (58 ± 24 vs. 86 ± 22%; $P = 0.11$). However, sputum MPO ($r = 0.36$; $P = 0.001$), LTB_4 ($r = 0.24$; $P = 0.03$), IL-8 ($r = 0.40$, $P < 0.001$), and neutrophil elastase ($r = 0.62$, $P < 0.001$) correlate well with sputum neutrophil counts, highlighting their biologic relevance.

Relationship between roflumilast treatment and clinical outcomes. Lung function, quality of life, and exercise tolerance were evaluated over the 12-week study period. Baseline values are reported in Table 1. As seen in Figure E3A, mean post-bronchodilator FEV_1 did not significantly improve compared with baseline or with placebo, but the 41-ml increase observed with roflumilast treatment was similar to what has previously been reported (37). Total SGRQ score (*see* Figure E3B), BCSS score (*see* Figure E3C), and 6MWD (*see* Figure E3D) showed trends toward improvement, although none reached statistical significance.

Safety and side-effect profile. The incidence of treatment-related adverse effects as assessed by the investigator was 45% in the roflumilast group and 25% in the placebo group ($P = 0.24$) (Table 2). Adverse effects of special interest that developed in the roflumilast group were gastrointestinal including nausea, diarrhea, and weight loss of 10 pounds (27%); upper respiratory infection (9%); cough (9%); and insomnia (9%). No significant adverse effect, death, or hospitalization occurred in the study. No adverse effects led to discontinuation of study drug.

Discussion

This study is the first evidence of roflumilast's ability to lower sputum

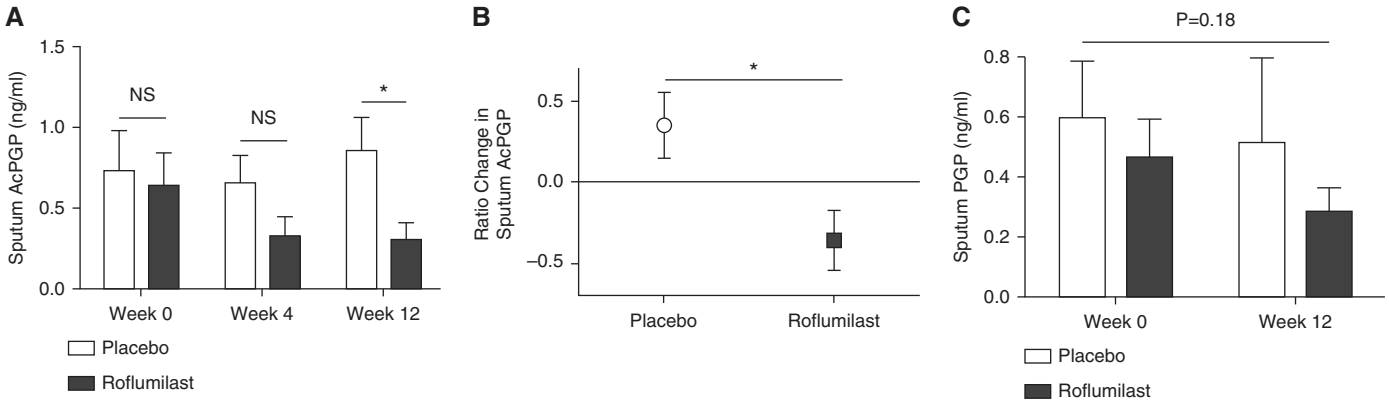

Figure 2. Roflumilast reduces sputum acetyl-proline-glycine-proline (AcPGP). Sputa were analyzed in randomized patients. (*A*) Roflumilast treatment results in lower AcPGP levels compared with placebo after 12 weeks of therapy. (*B*) Roflumilast reduces sputum AcPGP by >50%. Following 12 weeks of treatment with (*C*) roflumilast or placebo, there were no statistical changes in sputum proline-glycine-proline (PGP). *Open bars* and *circle* represent the placebo group (n = 16), and *solid bars* and *square* represent the roflumilast group (n = 11). Values expressed as mean ± SEM. *$P < 0.05$. NS = not significant.

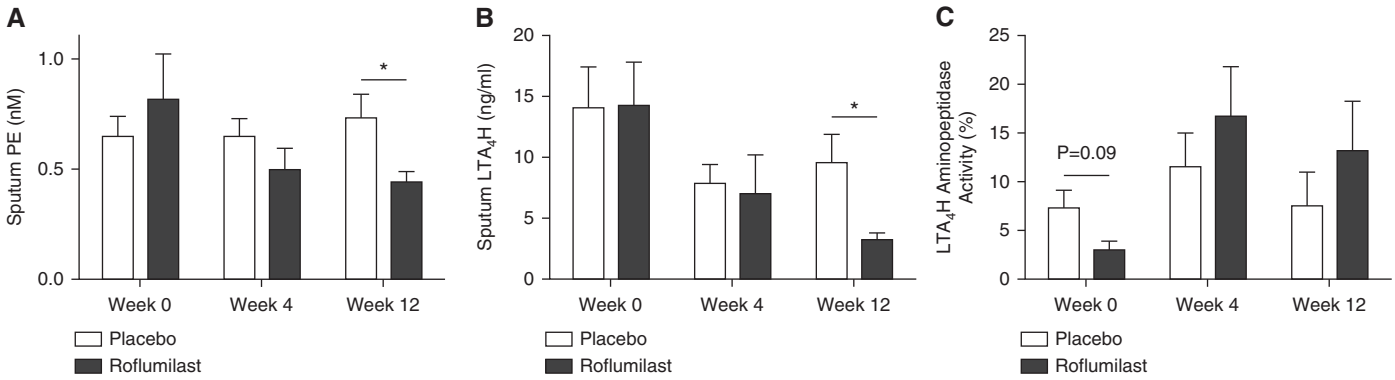

Figure 3. Roflumilast affects enzymes critical to the acetyl-proline-glycine-proline/proline-glycine-proline pathway. (*A*) Roflumilast decreases prolyl endopeptidase (PE), the enzyme critical in the generation of proline-glycine-proline. (*B*) Likewise, roflumilast treatment reduces leukotriene A_4 hydrolase (LTA_4H) amount in sputum compared with placebo. (*C*) Although there was an increase in LTA_4H aminopeptidase activity in roflumilast treatment after 12 weeks, there were no statistical differences in this group or placebo compared with baseline values. *Open bars* represent the placebo group (n = 16), and *solid bars* represent the roflumilast group (n = 11). Values expressed as mean ± SEM. $^*P < 0.05$.

AcPGP levels over a 12-week period that coincides with reductions in pulmonary inflammation in patients with moderate to severe COPD with chronic bronchitis. The reduction in sputum AcPGP by more than 50% achieved the primary end point of the study. We also demonstrate for the first time that reducing prolyl endopeptidase, the rate-limiting enzyme responsible for PGP/AcPGP release from collagen fragments, is affected by pharmacologic intervention. This is an example of translation of observations from *in vitro* and animal models to human subjects, highlighting the pathologic importance of AcPGP in the development of COPD (17) into a proof of concept clinical trial. Our findings suggest that the antiinflammatory effects of roflumilast are most pronounced in the lung and these effects are more pronounced than roflumilast's other properties as a bronchodilator and airway modulator. Roflumilast was well tolerated in this study.

The significant reduction in sputum AcPGP following 12 weeks of therapy with roflumilast occurred primarily because of inhibition of its generating pathway specifically by decreasing prolyl endopeptidase. We have previously demonstrated that prolyl endopeptidase is expressed on human macrophages, airway epithelial cells, and neutrophils, where it is increased 25-fold in subjects with COPD compared with healthy control subjects (38). We have also previously described the crucial role of prolyl endopeptidase in the *de novo* production of PGP and AcPGP from collagen fragments and shown that it is a viable therapeutic target using *in vitro* and animal models of COPD (25, 36, 39).

The current study highlights the importance of prolyl endopeptidase as a key enzyme in regulating neutrophilic inflammation in COPD and warrants further investigation. The 4.5-fold improvement in LTA_4H aminopeptidase function was not statistically significant. This may be caused in part by severe baseline disease in the patient population as evidenced by marked suppression of aminopeptidase function or may be a result of our small sample size. As we have previously shown, subjects with COPD have dramatically impaired aminopeptidase activity compared with healthy control and control smoking subjects (17).

In addition to the reductions in AcPGP, roflumilast also lowers other markers of inflammation. Here, total neutrophil count was reduced by 57%, IL-8 by 37%, and neutrophil elastase amount by 70%. These are comparable with the respective approximately 40%, approximately 38%, and approximately 30% reductions observed by Grootendorst and coworkers (12). We also found significant reductions in MPO and LTB_4, highlighting roflumilast's ability to reduce multiple markers of neutrophilic inflammation.

COPD exacerbations are a major target for novel therapeutic agents, including roflumilast and azithromycin. Although effective at reducing exacerbations, these drugs are given long-term and use is hampered by drug intolerance and other side effects. We have previously evaluated

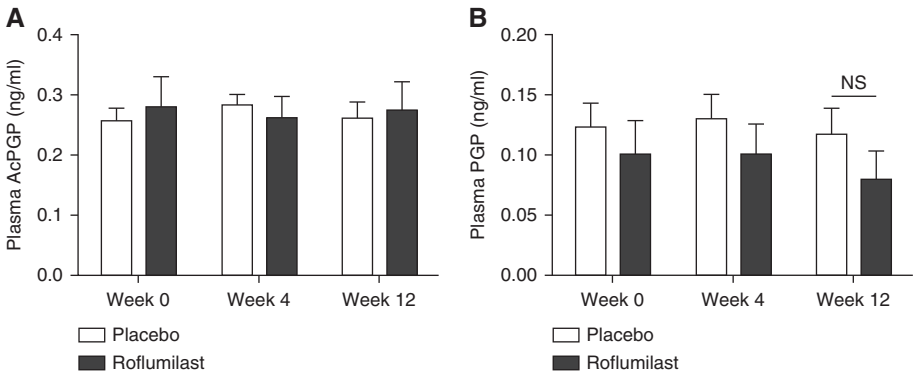

Figure 4. Roflumilast therapy does not affect systemic proline-glycine-proline (PGP). There were no statistical differences in plasma (*A*) acetyl-proline-glycine-proline (AcPGP) and (*B*) PGP, suggesting the antiinflammatory effect is most pronounced in the lung. *Open bars* represent the placebo group (n = 16), and *solid bars* represent the roflumilast group (n = 11). Values expressed as mean ± SEM. NS = not significant.

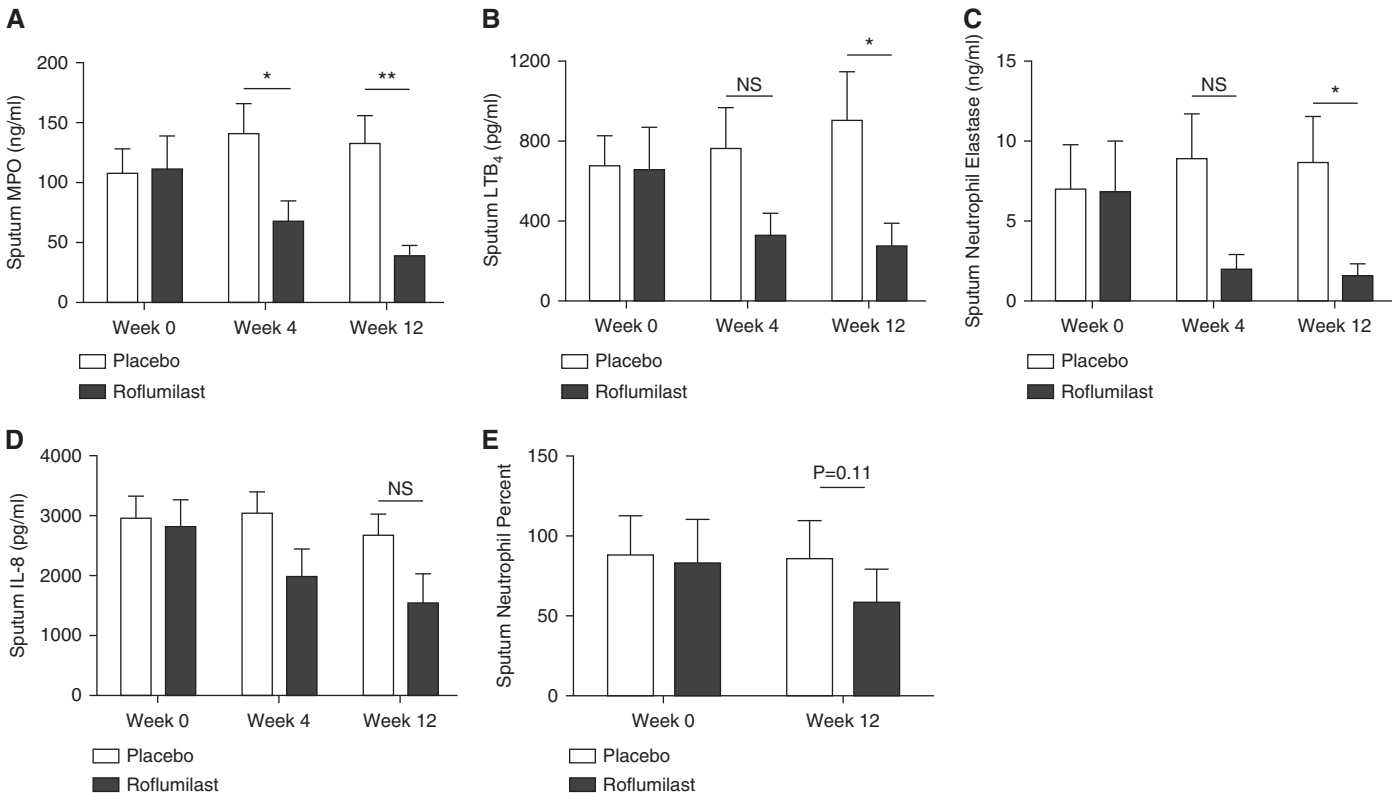

Figure 5. Roflumilast reduces other markers of pulmonary inflammation. (*A*) Myeloperoxidase (MPO) was significantly reduced at 4 and 12 weeks. Roflumilast use is associated with a reduction in (*B*) leukotriene B_4 (LTB_4) and (*C*) neutrophil elastase but not (*D*) IL-8. (*E*) Neutrophil counts were reduced, although no statistical difference was observed. *Open bars* represent the placebo group (n = 16), and *solid bars* represent the roflumilast group (n = 11). Values expressed as mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$. NS = not significant.

the association between sputum PGP, azithromycin use, and acute exacerbations (22). We found that azithromycin use for longer than 6 months was correlated to lower sputum PGP amounts. Here, we show that roflumilast treatment results in lowering of sputum AcPGP in 3 months, suggesting a more rapid and sustained treatment effect compared with azithromycin in severe COPD. Both the current study and the MACRO trial (40) enrolled COPD subjects with moderate to severe airflow obstruction that were at risk for COPD exacerbations. However, there were two major differences in the study populations: we specifically evaluated patients with chronic bronchitis in the current study, and subjects enrolled in the current study were more often current smokers (59% vs. ~22% in the MACRO trial). Taking these differences into context and supported by recent findings suggesting daily azithromycin therapy is most effective in nonsmoking subjects with COPD with milder airflow obstruction (41) may help explain observed differences in efficacy. Although a head-to-head trial is necessary to adequately distinguish the risk-benefit ratio for using roflumilast or azithromycin in different COPD populations, our data support the use of roflumilast as a treatment for moderate to severe COPD with chronic bronchitis by reducing pulmonary inflammation, even in the setting of ongoing cigarette use.

The present study is limited by its small sample size and limited follow-up. These limitations increase the likelihood that true

Table 2. Adverse Effects

	Placebo (*n = 16*)	Roflumilast (*n = 11*)
Any AE	4 (25%)	5 (45%)
Any significant AE	0	0
Treatment-related AE		
Any gastrointestinal	0	3 (27%)
Nausea	0	1 (9%)
Diarrhea	0	1 (9%)
Weight loss of 10 lb	0	1 (9%)
Any sinopulmonary	4 (25%)	2 (18%)
Upper respiratory infection	2 (13%)	1 (9%)
Cough	0	1 (9%)
Pleurisy	1 (6%)	0
Pneumonia	1 (6%)	0
Insomnia	0	1 (9%)

Definition of abbreviation: AE = adverse effect.
$P = 0.24$ for any AE differences between placebo and roflumilast groups.
All results are number of patients (%).

findings, such as improvement in FEV_1 or quality of life, were not observed. Despite these limitations, the important findings of reductions in sputum AcPGP and other markers of neutrophilic inflammation with chronic roflumilast treatment highlight the overall robust nature of these associations. An additional limitation was unequal randomization: a greater number of subjects randomized to placebo compared with roflumilast. Our randomization scheme used block randomization to account for smoking status and inhaled steroid use and this happened to result in the discrepancy. Finally, given the heterogeneity of COPD, novel computed tomography–based metrics are increasingly being used to subphenotype COPD, including chronic bronchitis (42, 43). We did not have access to computed tomography imaging in this study, but further studies should be performed to link the AcPGP-pathway with computed tomography findings of chronic bronchitis.

In summary, this study suggests for the first time that administration of roflumilast for 12 weeks can reduce lung inflammation through mechanistically inhibiting a pathway integral to the pathogenesis of COPD and implicated with COPD exacerbations. The beneficial clinical effect of treatment seen in patients with moderate-to-severe COPD at risk for acute exacerbations is most likely caused by these reductions in pulmonary prolyl endopeptidase, AcPGP, and neutrophilic inflammation. This study was powered on the basis of reductions in sputum AcPGP, therefore further adequately powered studies focusing on sputum and plasma AcPGP and clinical end points, such as exacerbation frequency or severity, are needed. Considering the present results together with previous findings with azithromycin, both PGP and AcPGP show promise as clinically relevant biomarkers for COPD. ■

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