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Sputum PGP is reduced by azithromycin treatment in COPD patients and correlates with exacerbations

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Key Words: COPD, neutrophil, exacerbation, PGP

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ABSTRACT

Rationale: Proline-glycine-proline (PGP), a neutrophil chemoattractant derived from the enzymatic breakdown of collagen, is elevated in sputum of patients with chronic obstructive pulmonary disease (COPD) and may contribute to disease progression. Whether sputum levels of PGP respond to therapy of COPD or predict outcomes is unknown.

Objectives: We conducted a study ancillary to a multicenter trial of the efficacy of azithromycin treatment for one year in preventing COPD exacerbations to test if sputum levels of PGP were altered by treatment or associated with exacerbation frequency.

Methods: We collected remnant sputa from trial participants and assayed them in a blinded fashion for PGP, myeloperoxidase and matrix metalloprotease (MMP)-9 and for the ability to generate PGP from collagen ex vivo. Once the parent trial was un-blinded, the results were correlated with use of azithromycin or placebo and exacerbations in participants.

Results: Azithromycin treatment significantly reduced sputum levels of PGP and myeloperoxidase in COPD patients, particularly with increased duration of therapy. We found no difference in sputum MMP-9 or PGP generation between subjects taking azithromycin or placebo. Sputum PGP levels were highest around the time of an exacerbation and declined with successful treatment.

Conclusions: These data support a role for PGP in the airway and parenchymal neutrophilic inflammation that drives COPD progression and exacerbations, and provide new information on the anti-inflammatory properties of macrolides. PGP may have potential as a target for novel anti-inflammatory therapies in COPD and as a biomarker for clinical trials.
ARTICLE SUMMARY

Strengths and limitations of this study

- This research was conducted ancillary to a multicenter, prospective, parallel group, placebo-controlled, double-blind study of the efficacy of azithromycin in the chronic, outpatient management of COPD.

- We demonstrate that chronic treatment with azithromycin reduces levels of PGP, a collagen-derived neutrophil chemoattractant, in sputum of COPD patients.

- This provides new information on the anti-inflammatory properties of macrolide antibiotics and supports a role for PGP in COPD pathogenesis and as a biomarker in clinical trials.

- The number of subjects included in this study is small as sputum samples were available only from a minority of participants in the parent trial.
INTRODUCTION

Proline-Glycine-Proline (PGP) is a tripeptide generated from the breakdown of extracellular matrix collagen and is specifically chemotactic for neutrophils in vitro and in vivo [1]. PGP exerts its chemotactic effect through sequence homology with a key motif found in ELR+ CXC chemokines and binds to their receptors, CXCR1 and CXCR2 [1]. PGP is generated from native collagen by the action of neutrophil-derived matrix metalloproteinases (MMP’s) 8 and 9, which cleave collagen into oligopeptides, followed by a secondary cleavage by prolyl endopeptidase [2], a neuronal enzyme implicated in hypertension and neuropeptide processing. Prolyl endopeptidase is also present in neutrophils where it likely participates in generation of PGP [3]. PGP is broken down in the lung by the aminopeptidase activity of leukotriene A4 hydrolase (LTA4H), an enzyme hitherto recognized only as proinflammatory through generation of leukotriene B4 [4]. Consequently, acute PGP-driven neutrophilic inflammation may be terminated by LTA4H. Cigarette smoke can selectively inactivate the aminopeptidase, but not the hydrolase, activity of LTA4H, thus allowing accumulation of PGP, while promoting neutrophilic inflammation [4].

We have previously demonstrated that PGP is a potential sputum and serum biomarker in chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), and chronic lung transplant rejection and contributes to neutrophilic airway inflammation in these diseases [2 5 6]. Sputa from patients with CF and COPD generate PGP from collagen ex vivo [2 5]. Levels of PGP in CF sputum are higher during exacerbations and decline with successful treatment and resolution [2]. Whether PGP levels are modifiable by outpatient therapy of lung diseases or whether such
changes can positively impact clinical outcomes has not been tested. This is important, as no biomarker has yet been identified that can reliably aid in diagnosis and phenotyping of COPD or serve as a surrogate endpoint for clinical trials. Identifying such biomarkers is a key priority [7 8].

Existing pharmacotherapies do not alter the neutrophilic airway and parenchymal inflammation that are at the heart of disease progression in COPD and consequently do not prevent loss of lung function. Accordingly, there is a need for new therapies. One candidate is azithromycin, a macrolide antibiotic with anti-inflammatory properties. Azithromycin and other macrolides have been proven beneficial in chronic, neutrophilic airway diseases, such as bronchiectasis, diffuse panbronchiolitis, and CF [9 10]. These effects are believed to be due, in part, to an as-yet unexplained anti-inflammatory activity and not just suppression of bacterial growth [11].

The National Heart Lung and Blood Institute (NHLBI) recently sponsored a multicenter trial of azithromycin in stable outpatients with COPD (the Macrolide trial) [12]. As macrolides have been demonstrated to reduce MMP activity in lung diseases, including COPD [11 13 14], we hypothesized that beneficial effects of azithromycin might be mediated through reducing PGP. Accordingly we conducted a study ancillary to the Macrolide trial in which sputum collected from participants for microbial analysis was analyzed for PGP and the ability to generate PGP from collagen. Once the code for the parent trial was broken, we correlated PGP levels with use of azithromycin or placebo and clinical response. The results are herein reported.
METHODS

Patient recruitment

This research was carried out as an ancillary study to the Macrolide trial of the NHLBI COPD Clinical Research Network. This was a prospective, parallel group, placebo-controlled, double-blind study of the efficacy of azithromycin in the chronic, outpatient management of COPD with a primary outcome of time to first exacerbation [12]. COPD patients, selected to be at high risk of acute exacerbation, were randomized to receive either azithromycin 250mg daily or placebo for twelve months. Nasopharyngeal swabs were obtained from participants at various points during the trial (at randomization, after one, three, six, nine and twelve months of treatment and one month after completion) to screen for colonization by macrolide-resistant pathogens. In a limited number of subjects, sputum samples were also obtained for microbial analysis. For the current study, we obtained remnant sputum from these subjects for measurement of biomarkers. Approval for this study was obtained from the Institutional Review Board of the University of Alabama at Birmingham and the coordinating center of the Macrolide trial (University of Minnesota, Rochester, MN).

Measurement of PGP, myeloperoxidase and MMP-9 in sputum

Sputum was diluted 1:1 with normal saline, 10 kDa filtered, washed with 40 µl of 1 N HCl and assayed for PGP by electrospray ionization-liquid chromatography-mass spectrometry/mass spectrometry (ESI-LC-MS/MS) as previously described [2 5]. The detection limit was 10 pg/ml.
Myeloperoxidase was measured in sputum using a commercially available activity assay (Calbiochem, San Diego, California, USA). Samples and standards were added to wells coated with a polyclonal antibody to human myeloperoxidase and incubated for two hours. The detection reagent (tetramethylbenzidine and hydrogen peroxidase) was added for one hour and absorbance read at 450 nm. Activity was converted to ng/ml active myeloperoxidase using a standard curve.

The concentration of total and pro-MMP-9 was determined in sputum using commercially available ELISA kits (R&D Systems, Minneapolis, MN). Samples and standards were added to wells coated with a monoclonal antibody to MMP-9 and incubated for two hours at room temperature. A fluorogenic substrate (Fluor-Pro-Leu-Gly-Leu-Ala-Arg-NH₂) was added and the plate incubated for 18 hrs at 37°C. Activity was quantified using a spectrophotometer with excitation and emission wavelengths of 320 and 450 nm respectively and converted to ng/ml active MMP-9 using a standard curve.

**Generation of PGP ex vivo**

100 μl of sputum was incubated with extensively dialyzed (against dH₂O with pH adjusted to 4.5 by addition of glacial acetic acid) intact type I or II collagen (10 μl, 1 mg/ml, Sigma-Aldrich, St. Louis, MO) for 24 hours at 37°C and 5% CO₂. The samples were filtered through a 10 kDa filter, washed with 40 μl of 1 N HCl and analyzed using ESI- LC-MS/MS for levels of PGP. Amounts of PGP generated by each sample were determined by comparison with PGP generated by sputum incubated without collagen.
Data analysis

Sputum samples were identified as coming from participants taking azithromycin or placebo once the Macrolide trial was competed and un-blinded. Number of exacerbations for each participant and the date of onset of symptoms for each exacerbation were recorded. Data are presented as means ± SEM. Sputum biomarker levels and activities were compared between groups using Student’s t-test. All p-values <0.05 were considered significant.

RESULTS

Azithromycin reduces sputum PGP and myeloperoxidase compared with placebo in a time-dependent fashion

We obtained a total of 46 sputum samples from COPD patients recruited to the Macrolide trial and assayed them in a blinded fashion for levels of PGP. Un-blinding of the parent trial revealed there were 18 sputum samples from 13 placebo-treated subjects and 14 sputum samples from 8 azithromycin-treated subjects collected at months one through twelve of treatment. Sputum PGP levels were significantly higher in subjects taking placebo compared to those taking azithromycin during the trial (5.15 ± 1.54 vs. 2.27 ± 0.87 ng/ml, p<0.05, Figure 1). The difference in sputum PGP was greatest at months nine and twelve, indicating a cumulative effect of azithromycin treatment (Figure 2).
The difference in sputum myeloperoxidase activity between subjects taking placebo or azithromycin over the twelve months of the trial was not statistically significant (3391 ± 353 vs. 2884 ± 349 ng/ml, p=0.16). However, sputum myeloperoxidase was lower in subjects taking azithromycin from months six through twelve (3321 ± 430 vs. 2398 ± 290 ng/ml, p<0.05), indicating a cumulative effect of azithromycin treatment on neutrophilic airway inflammation.

There was no difference between subjects taking placebo or azithromycin in sputum MMP-9 activity (11.18 ± 3.98 vs. 8.39 ± 2.42 ng/ml, NS) or PGP generation from collagen ex vivo (3.28 ± 2.25 vs. 2.94 ± 1.73 ng/ml, NS) during the trial.

**Fall in sputum PGP is associated with reduced exacerbation frequency in COPD patients**

In the Macrolide trial, treatment with azithromycin significantly reduced exacerbation frequency in high risk COPD patients compared to placebo (1.48 vs. 1.83 exacerbations per patient year, p=0.01) and increased the time to first exacerbation [12]. We saw a similar reduction in exacerbation frequency in subjects taking azithromycin in our ancillary study although this was not significant due to the small size of our study group (0.91 ± 0.37 vs. 1.93 ± 0.62 exacerbations per patient year, p=0.08).

**Sputum PGP rises during COPD exacerbations and declines with resolution**

We obtained the dates of exacerbations experienced by our subjects during the Macrolide trial and correlated sputum PGP with time in days from the closest exacerbation. This resulted in an interesting pattern that suggested that PGP levels precipitously rise in advance of an exacerbation and rapidly decline thereafter (Figure 3). Indeed, sputum PGP was higher around
the time of an exacerbation (18 ± 4 days before or after, 12.8 ± 4.53 ng PGP/ml, n = 9) compared to long before (103 ± 37 days, 1.10 ± 0.411 ng PGP/ml, n = 6, p<0.02) or after an exacerbation (141 ± 11 days, 2.15 ± 0.80 ng PGP/ml, n = 11, p<0.02, Figure 3 inset). There was a trend toward higher sputum PGP after an exacerbation compared to before (p=0.133).

**DISCUSSION**

PGP is a neutrophil chemoattractant in vitro and in vivo and contributes to airway inflammation in chronic lung diseases with a prominent neutrophil component including COPD and CF [2 5 6]. Chronic instillation of PGP into mouse lungs results in emphysema and right ventricular hypertrophy [1] and inhibition of PGP by a complementary peptide (arginine-threonine-arginine) reduces acute cigarette smoke-induced neutrophil influx into mouse lungs and prevents emphysema and right ventricular hypertrophy in a mouse model of COPD [15 16]. However, direct evidence implicating PGP in COPD pathogenesis in humans is lacking. In the limited number of sputum samples available to us due to the design of the Macrolide trial, we demonstrate that PGP is reduced in the airways of COPD patients taking azithromycin, particularly with longer duration of therapy, and is accompanied by reduced neutrophil burden, measured as sputum myeloperoxidase. Reduction in sputum PGP is accompanied by a positive clinical response to azithromycin (reduced exacerbation frequency). Our subgroup had a similar therapeutic response to that seen in the parent trial, suggesting that our findings could be generalized to the entire Macrolide group.
Although seen with only a few specimens, sputum PGP was strikingly elevated as long as 35 days before the onset of symptoms of an acute exacerbation of COPD and briefly afterwards. It declined sharply with successful treatment and resolution (Figure 3). Seemungal et al described a seven day prodrome where respiratory symptoms worsened before the onset of an exacerbation but failed to predict exacerbations with spirometry [17]. Our data suggest that airway inflammation may worsen weeks in advance of this and that PGP may predict the onset of an exacerbation long enough before symptoms appear to permit early intervention.

Although many biomarkers of COPD have been described, to our knowledge none has been shown to herald exacerbations [18]. The persistence of elevated sputum PGP long after exacerbations in some individuals may contribute to continuing neutrophilic airway inflammation and promote the “frequent exacerbator” phenotype [19-21].

We cannot determine from our data whether the reduction in exacerbations due to azithromycin was secondary to reduced sputum PGP or whether the reduction in sputum PGP was due to fewer exacerbations in azithromycin-treated subjects. Answering this question definitively would require a larger, longitudinal study of PGP in COPD patients. Nevertheless, these data support the idea that PGP plays a significant role in COPD pathogenesis, particularly exacerbations, and may provide important clues as to the mechanism of azithromycin’s anti-inflammatory properties. PGP predicted a positive response to azithromycin within six months of starting therapy and was superior in this respect to other biomarkers studied as part of the Macrolide trial [22].
We are uncertain as to the mechanism of PGP reduction by azithromycin. Macrolide antibiotics have been reported to have numerous anti-inflammatory properties, including inhibiting MMP activity [11 13 14]. As MMP-8 and 9 are essential to extracellular PGP generation from collagen by neutrophils [2], this would provide an attractive potential mechanism. However, we could find no difference in sputum MMP-9 activity between treatment groups. We did not examine other MMP's which we have previously implicated in PGP generation, such as MMP-1 and MMP-8 [2 5]. We measured PGP generation ex vivo from collagen by sputum but could find no difference between treatment groups. Subsequent to the current study, we have identified a neutrophil-derived enzyme, LTA₄H, which generates leukotriene B₄ and rapidly degrades PGP [4]. We have recently found that LTA₄H is markedly elevated in COPD sputum compared to controls [23]. Breakdown of PGP by LTA₄H in our sputum samples could have confounded the PGP generation assay. As PGP is derived from neutrophil activity, reduced sputum PGP might reflect reduced recruitment of neutrophils to the airways due to other effects of azithromycin. The decline in sputum PGP was greater than the decline in myeloperoxidase, suggesting that PGP may have other effects in COPD aside from recruiting neutrophils. Addressing these questions was outside the scope of this study.

In conclusion, the results of our study support the idea that PGP plays an important role in COPD pathogenesis and provide clues as to the anti-inflammatory mechanism of azithromycin. PGP shows promise as a predictor of acute exacerbations and may serve as a useful biomarker for response to therapy in future studies of COPD and other chronic, neutrophilic lung diseases.
ACKNOWLEDGMENTS

We would like to thank the NHLBI COPD clinical research network for their assistance in providing sputum samples and clinical data for this study.

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AUTHORS’ CONTRIBUTIONS

PO’R designed the study, acquired and interpreted data and drafted the manuscript. PLJ and JMW acquired and interpreted data. MTD and PDS provided sputum samples. JEB designed the study and supervised the project. All authors contributed to revision and final approval of the manuscript.

COMPETING INTERESTS

The authors have no competing interests.

DATA SHARING

There is no additional data available.
ETHICS

Permission to conduct this research came from the Institutional Review Board of the University of Alabama at Birmingham and from the steering committee of the NHLBI COPD clinical research network who conducted the Macrolide trial.
REFERENCES


23. Wells JM, Jackson PL, O'Reilly PJ, Blalock JE. Selective Inhibition Of Leukotriene A4 Hydrolase Aminopeptidase Activity Occurs In COPD And Reflects Clinical Outcomes. American journal of respiratory and critical care medicine 2012;185:A1422
FIGURE LEGENDS

Figure 1. Azithromycin treatment decreases PGP levels in sputum of COPD patients compared with placebo. Inset: values represent the mean ± SEM PGP concentrations for sputa (*p<0.05 vs. placebo).

Figure 2. Sputum PGP levels decline with increased duration of azithromycin therapy. PGP levels were significantly higher in subjects treated with placebo compared to azithromycin at months nine and twelve of therapy (*p<0.02) but not at months one through six (p=0.32).

Figure 3. Sputum PGP levels are highest around the time of a COPD exacerbation. Values represent PGP levels at various days relative to the onset of an exacerbation (time 0). Inset: values represent the mean ± SEM PGP concentrations for sputa collected 103 ± 37 days before an exacerbation (pre), 18 ± 4 days before or after an exacerbation (peri) or 141 ± 11 days after an exacerbation (post). *p<0.02 (pre vs. peri), †p<0.02 (post vs. peri)
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RESULTS

Azithromycin reduces sputum PGP and myeloperoxidase compared with placebo in a time-dependent fashion

We obtained a total of 46 sputum samples from COPD patients recruited to the Macrolide trial and assayed them in a blinded fashion for levels of PGP. Un-blinding of the parent trial revealed there were 18 sputum samples from 13 placebo-treated subjects and 14 sputum samples from 8 azithromycin-treated subjects collected at months one through twelve of treatment. Clinical characteristics of our study subjects were similar to those of the parent trial (age = 67.6 ± 8.4 years, 30% female, post-bronchodilator FEV\textsubscript{1} = 1.36 ± 0.47L, mean ± SD).

Sputum PGP levels were significantly higher in subjects taking placebo compared to those taking azithromycin during the trial (5.15 ± 1.54 vs. 2.27 ± 0.87 ng/ml, p<0.05, Figure 1). The difference in sputum PGP was greatest at months nine and twelve, indicating a cumulative effect of azithromycin treatment (Figure 2). Given the limited data set, effects of azithromycin
treatment on PGP levels are reported for groups. However, there was a progressive decline in
sputum PGP with duration of treatment in 5 of 6 azithromycin-treated subjects whose sputum
was assayed at more than one time-point.

The difference in sputum myeloperoxidase activity between subjects taking placebo or
azithromycin over the twelve months of the trial was not statistically significant (3391 ± 353 vs.
2884 ± 349 ng/ml, p=0.16). However, sputum myeloperoxidase was lower in subjects taking
azithromycin from months six through twelve (3321 ± 430 vs. 2398 ± 290 ng/ml, p<0.05),
indicating a cumulative effect of azithromycin treatment on neutrophilic airway inflammation.

There was no difference between subjects taking placebo or azithromycin in sputum MMP-9
activity (11.18 ± 3.98 vs. 8.39 ± 2.42 ng/ml, NS) or PGP generation from collagen ex vivo (3.28 ±
2.25 vs. 2.94 ± 1.73 ng/ml, NS) during the trial.

**Fall in sputum PGP is associated with reduced exacerbation frequency in COPD patients**

In the Macrolide trial, treatment with azithromycin significantly reduced exacerbation
frequency in high risk COPD patients compared to placebo (1.48 vs. 1.83 exacerbations per
patient year, p=0.01) and increased the time to first exacerbation [12]. We saw a similar
reduction in exacerbation frequency in subjects taking azithromycin in our ancillary study
although this was not significant due to the small size of our study group (0.91 ± 0.37 vs. 1.93 ±
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Sputum PGP rises during COPD exacerbations and declines with resolution

We obtained the dates of exacerbations experienced by our subjects during the Macrolide trial, including both placebo and azithromycin-treated groups, and correlated sputum PGP with time in days from the closest exacerbation. This resulted in an interesting pattern that suggested that PGP levels precipitously rise in advance of an exacerbation and rapidly decline thereafter (Figure 3). Indeed, sputum PGP was higher around the time of an exacerbation (18 ± 4 days before or after, 12.8 ± 4.53 ng PGP/ml, n = 9) compared to long before (103 ± 37 days, 1.10 ± 0.411 ng PGP/ml, n = 6, p<0.02) or after an exacerbation (141 ± 11 days, 2.15 ± 0.80 ng PGP/ml, n = 11, p<0.02, Figure 3 inset). There was a trend toward higher sputum PGP after an exacerbation compared to before (p=0.133).

DISCUSSION

PGP is a neutrophil chemoattractant in vitro and in vivo and contributes to airway inflammation in chronic lung diseases with a prominent neutrophil component including COPD and CF [2 5 6]. Chronic instillation of PGP into mouse lungs results in emphysema and right ventricular hypertrophy [1] and inhibition of PGP by a complementary peptide (arginine-threonine-arginine) reduces acute cigarette smoke-induced neutrophil influx into mouse lungs and prevents emphysema and right ventricular hypertrophy in a mouse model of COPD [15 16]. However, direct evidence implicating PGP in COPD pathogenesis in humans is lacking. In the limited number of sputum samples available to us due to the design of the Macrolide trial, we demonstrate that PGP is reduced in the airways of COPD patients taking azithromycin,
particularly with longer duration of therapy, and is accompanied by reduced neutrophil burden, measured as sputum myeloperoxidase. Reduction in sputum PGP is accompanied by a positive clinical response to azithromycin (reduced exacerbation frequency). Our subgroup had a similar therapeutic response to that seen in the parent trial, suggesting that our findings could be generalized to the entire Macrolide group.

Although seen with only a few specimens, sputum PGP was strikingly elevated as long as 35 days before the onset of symptoms of an acute exacerbation of COPD and briefly afterwards. It declined sharply with successful treatment and resolution (Figure 3). Seemungal et al described a seven day prodrome where respiratory symptoms worsened before the onset of an exacerbation but failed to predict exacerbations with spirometry [17]. Our data suggest that airway inflammation may worsen weeks in advance of this and that PGP may predict the onset of an exacerbation long enough before symptoms appear to permit early intervention.

Although many biomarkers of COPD have been described, to our knowledge none has been shown to herald exacerbations [18]. The persistence of elevated sputum PGP long after exacerbations in some individuals may contribute to continuing neutrophilic airway inflammation and promote the “frequent exacerbator” phenotype [19-21].

We cannot determine from our data whether the reduction in exacerbations due to azithromycin was secondary to reduced sputum PGP or whether the reduction in sputum PGP was due to fewer exacerbations in azithromycin-treated subjects. Answering this question definitively would require a larger, longitudinal study of PGP in COPD patients. Nevertheless, these data support the idea that PGP plays a significant role in COPD pathogenesis, particularly...
exacerbations, and may provide important clues as to the mechanism of azithromycin’s anti-inflammatory properties. PGP predicted a positive response to azithromycin within six months of starting therapy and was superior in this respect to other biomarkers studied as part of the Macrolide trial [22].

We are uncertain as to the mechanism of PGP reduction by azithromycin. Macrolide antibiotics have been reported to have numerous anti-inflammatory properties, including inhibiting MMP activity [11 13 14]. As MMP-8 and 9 are essential to extracellular PGP generation from collagen by neutrophils [2], this would provide an attractive potential mechanism. However, we could find no difference in sputum MMP-9 activity between treatment groups. We did not examine other MMP’s which we have previously implicated in PGP generation, such as MMP-1 and MMP-8 [2 5]. We measured PGP generation ex vivo from collagen by sputum but could find no difference between treatment groups. Subsequent to the current study, we have identified a neutrophil-derived enzyme, LTA₄H, which generates leukotriene B₄ and rapidly degrades PGP [4]. We have recently found that LTA₄H is markedly elevated in COPD sputum compared to controls [23]. Breakdown of PGP by LTA₄H in our sputum samples could have confounded the PGP generation assay. As PGP is derived from neutrophil activity, reduced sputum PGP might reflect reduced recruitment of neutrophils to the airways due to other effects of azithromycin. The decline in sputum PGP was greater than the decline in myeloperoxidase, suggesting that PGP may have other effects in COPD aside from recruiting neutrophils. Addressing these questions was outside the scope of this study.
In conclusion, the results of our study support the idea that PGP plays an important role in COPD pathogenesis and provide clues as to the anti-inflammatory mechanism of azithromycin. PGP shows promise as a predictor of acute exacerbations and may serve as a useful biomarker for response to therapy in future studies of COPD and other chronic, neutrophilic lung diseases.
ACKNOWLEDGMENTS

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FUNDING

Funding for this research came from grants HL090999, HL110950, HL077783 (to JEB) and HL092296 (to PO’R) from the National Heart and Blood Institute of the National Institutes of Health. The sponsor had no role in the study design, in the collection, analysis and interpretation of data, in the writing of the report or the decision to submit for publication.

AUTHORS’ CONTRIBUTIONS

PO’R designed the study, acquired and interpreted data and drafted the manuscript. PLJ and JMW acquired and interpreted data. MTD and PDS provided sputum samples. JEB designed the study and supervised the project. All authors contributed to revision and final approval of the manuscript.

COMPETING INTERESTS

The authors have no competing interests.

DATA SHARING

No additional unpublished data are available from this study.
ETHICS

Ethics approval for this study was provided by the Institutional Review Board at the University of Alabama at Birmingham. Approval for this study was also provided by the Steering Committee of the NHLBI COPD Clinical Research Network which conducted the original trial. This study was not part of the original trial protocol and was developed post hoc. Registration for the original trial is at clinicaltrials.gov, identifier: NCT00325897.
REFERENCES


FIGURE LEGENDS

Figure 1. Azithromycin treatment decreases PGP levels in sputum of COPD patients compared with placebo. Inset: values represent the mean ± SEM PGP concentrations for sputa (*p<0.05 vs. placebo).

Figure 2. Sputum PGP levels decline with increased duration of azithromycin therapy. PGP levels were significantly higher in subjects treated with placebo compared to azithromycin at months nine and twelve of therapy (*p<0.02) but not at months one through six (p=0.32).

Figure 3. Sputum PGP levels are highest around the time of a COPD exacerbation. Values represent PGP levels at various days relative to the onset of an exacerbation (time 0). Inset: values represent the mean ± SEM PGP concentrations for sputa collected 103 ± 37 days before an exacerbation (pre), 18 ± 4 days before or after an exacerbation (peri) or 141 ± 11 days after an exacerbation (post). *p<0.02 (pre vs. peri), #p<0.02 (post vs. peri)
Sputum PGP is reduced by azithromycin treatment in COPD patients and correlates with exacerbations

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ABSTRACT

Rationale: Proline-glycine-proline (PGP), a neutrophil chemoattractant derived from the enzymatic breakdown of collagen, is elevated in sputum of patients with chronic obstructive pulmonary disease (COPD) and may contribute to disease progression. Whether sputum levels of PGP respond to therapy of COPD or predict outcomes is unknown.

Objectives: We conducted a study ancillary to a multicenter trial of the efficacy of azithromycin treatment for one year in preventing COPD exacerbations to test if sputum levels of PGP were altered by treatment or associated with exacerbation frequency.

Methods: We collected remnant sputa from trial participants and assayed them in a blinded fashion for PGP, myeloperoxidase and matrix metalloprotease (MMP)-9 and for the ability to generate PGP from collagen ex vivo. Once the parent trial was un-blinded, the results were correlated with use of azithromycin or placebo and exacerbations in participants.

Results: Azithromycin treatment significantly reduced sputum levels of PGP and myeloperoxidase in COPD patients, particularly with increased duration of therapy. We found no difference in sputum MMP-9 or PGP generation between subjects taking azithromycin or placebo. Sputum PGP levels were highest around the time of an exacerbation and declined with successful treatment.

Conclusions: These data support a role for PGP in the airway and parenchymal neutrophilic inflammation that drives COPD progression and exacerbations, and provide new information on the anti-inflammatory properties of macrolides. PGP may have potential as a target for novel anti-inflammatory therapies in COPD and as a biomarker for clinical trials.
ARTICLE SUMMARY

Strengths and limitations of this study

- This research was conducted ancillary to a multicenter, prospective, parallel group, placebo-controlled, double-blind study of the efficacy of azithromycin in the chronic, outpatient management of COPD.

- We demonstrate that chronic treatment with azithromycin reduces levels of PGP, a collagen-derived neutrophil chemoattractant, in sputum of COPD patients.

- This provides new information on the anti-inflammatory properties of macrolide antibiotics and supports a role for PGP in COPD pathogenesis and as a biomarker in clinical trials.

- The number of subjects included in this study is small as sputum samples were available only from a minority of participants in the parent trial.
INTRODUCTION

Proline-Glycine-Proline (PGP) is a tripeptide generated from the breakdown of extracellular matrix collagen and is specifically chemotactic for neutrophils in vitro and in vivo [1]. PGP exerts its chemotactic effect through sequence homology with a key motif found in ELR^+ CXC chemokines and binds to their receptors, CXCR1 and CXCR2 [1]. PGP is generated from native collagen by the action of neutrophil-derived matrix metalloproteinases (MMP’s) 8 and 9, which cleave collagen into oligopeptides, followed by a secondary cleavage by prolyl endopeptidase [2], a neuronal enzyme implicated in hypertension and neuropeptide processing. Prolyl endopeptidase is also present in neutrophils where it likely participates in generation of PGP [3]. PGP is broken down in the lung by the aminopeptidase activity of leukotriene A_4 hydrolase (LTA_4H), an enzyme hitherto recognized only as proinflammatory through generation of leukotriene B_4 [4]. Consequently, acute PGP-driven neutrophilic inflammation may be terminated by LTA_4H. Cigarette smoke can selectively inactivate the aminopeptidase, but not the hydrolase, activity of LTA_4H, thus allowing accumulation of PGP, while promoting neutrophilic inflammation [4].

We have previously demonstrated that PGP is a potential sputum and serum biomarker in chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), and chronic lung transplant rejection and contributes to neutrophilic airway inflammation in these diseases [2 5 6]. Sputa from patients with CF and COPD generate PGP from collagen ex vivo [2 5]. Levels of PGP in CF sputum are higher during exacerbations and decline with successful treatment and resolution [2]. Whether PGP levels are modifiable by outpatient therapy of lung diseases or whether such
changes can positively impact clinical outcomes has not been tested. This is important, as no biomarker has yet been identified that can reliably aid in diagnosis and phenotyping of COPD or serve as a surrogate endpoint for clinical trials. Identifying such biomarkers is a key priority [7 8].

Existing pharmacotherapies do not alter the neutrophilic airway and parenchymal inflammation that are at the heart of disease progression in COPD and consequently do not prevent loss of lung function. Accordingly, there is a need for new therapies. One candidate is azithromycin, a macrolide antibiotic with anti-inflammatory properties. Azithromycin and other macrolides have been proven beneficial in chronic, neutrophilic airway diseases, such as bronchiectasis, diffuse panbronchiolitis, and CF [9 10]. These effects are believed to be due, in part, to an as-yet unexplained anti-inflammatory activity and not just suppression of bacterial growth [11].

The National Heart Lung and Blood Institute (NHLBI) recently sponsored a multicenter trial of azithromycin in stable outpatients with COPD (the Macrolide trial) [12]. As macrolides have been demonstrated to reduce MMP activity in lung diseases, including COPD [11 13 14], we hypothesized that beneficial effects of azithromycin might be mediated through reducing PGP. Accordingly we conducted a study ancillary to the Macrolide trial in which sputum collected from participants for microbial analysis was analyzed for PGP and the ability to generate PGP from collagen. Once the code for the parent trial was broken, we correlated PGP levels with use of azithromycin or placebo and clinical response. The results are herein reported.
METHODS

Patient recruitment

This research was carried out as an ancillary study to the Macrolide trial of the NHLBI COPD Clinical Research Network (CRN). This was a prospective, parallel group, placebo-controlled, double-blind study of the efficacy of azithromycin in the chronic, outpatient management of COPD with a primary outcome of time to first exacerbation [12]. COPD patients, selected to be at high risk of acute exacerbation, were randomized to receive either azithromycin 250mg daily or placebo for twelve months. Nasopharyngeal swabs were obtained from participants at various points during the trial (at randomization, after one, three, six, nine and twelve months of treatment and one month after completion) to screen for colonization by macrolide-resistant pathogens. In a limited number of subjects, sputum samples were also obtained for microbial analysis. For the current study, we obtained remnant sputum from these subjects for measurement of biomarkers. Approval for this ancillary study was obtained from the Institutional Review Board of the University of Alabama at Birmingham and from the coordinating center of the CRN (University of Minnesota, Rochester, MN) which conducted the parent trial.

Measurement of PGP, myeloperoxidase and MMP-9 in sputum

Sputum was diluted 1:1 with normal saline, 10 kDa filtered, washed with 40 μl of 1 N HCl and assayed for PGP by electrospray ionization-liquid chromatography-mass spectrometry/mass spectrometry (ESI-LC-MS/MS) as previously described [25]. The detection limit was 10 pg/ml.
Myeloperoxidase was measured in sputum using a commercially available activity assay (Calbiochem, San Diego, California, USA). Samples and standards were added to wells coated with a polyclonal antibody to human myeloperoxidase and incubated for two hours. The detection reagent (tetramethylbenzidine and hydrogen peroxidase) was added for one hour and absorbance read at 450 nm. Activity was converted to ng/ml active myeloperoxidase using a standard curve.

The concentration of total and pro-MMP-9 was determined in sputum using commercially available ELISA kits (R&D Systems, Minneapolis, MN). Samples and standards were added to wells coated with a monoclonal antibody to MMP-9 and incubated for two hours at room temperature. A fluorogenic substrate (Fluor-Pro-Leu-Gly-Leu-Ala-Arg-NH$_2$) was added and the plate incubated for 18 hrs at 37°C. Activity was quantified using a spectrophotometer with excitation and emission wavelengths of 320 and 450 nm respectively and converted to ng/ml active MMP-9 using a standard curve.

**Generation of PGP ex vivo**

100 µl of sputum was incubated with extensively dialyzed (against dH$_2$O with pH adjusted to 4.5 by addition of glacial acetic acid) intact type I or II collagen (10 µl, 1 mg/ml, Sigma-Aldrich, St. Louis, MO) for 24 hours at 37°C and 5% CO$_2$. The samples were filtered through a 10 kDa filter, washed with 40 µl of 1 N HCl and analyzed using ESI- LC-MS/MS for levels of PGP. Amounts of PGP generated by each sample were determined by comparison with PGP generated by sputum incubated without collagen.
Data analysis

Sputum samples were identified as coming from participants taking azithromycin or placebo once the Macrolide trial was competed and un-blinded. Number of exacerbations for each participant and the date of onset of symptoms for each exacerbation were recorded. Data are presented as means ± SEM. Sputum biomarker levels and activities were compared between groups using Student’s t-test. All p-values <0.05 were considered significant.

RESULTS

Azithromycin reduces sputum PGP and myeloperoxidase compared with placebo in a time-dependent fashion

We obtained a total of 46 sputum samples from COPD patients recruited to the Macrolide trial and assayed them in a blinded fashion for levels of PGP. Un-blinding of the parent trial revealed there were 18 sputum samples from 13 placebo-treated subjects and 14 sputum samples from 8 azithromycin-treated subjects collected at months one through twelve of treatment. Clinical characteristics of our study subjects were similar to those of the parent trial (age = 67.6 ± 8.4 years, 30% female, post-bronchodilator FEV₁ = 1.36 ± 0.47L, mean ± SD).

Sputum PGP levels were significantly higher in subjects taking placebo compared to those taking azithromycin during the trial (5.15 ± 1.54 vs. 2.27 ± 0.87 ng/ml, p<0.05, Figure 1). The difference in sputum PGP was greatest at months nine and twelve, indicating a cumulative effect of azithromycin treatment (Figure 2). Given the limited data set, effects of azithromycin
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ACKNOWLEDGMENTS

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FUNDING

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AUTHORS’ CONTRIBUTIONS

PO’R designed the study, acquired and interpreted data and drafted the manuscript. PLJ and JMW acquired and interpreted data. MTD and PDS provided sputum samples. JEB designed the study and supervised the project. All authors contributed to revision and final approval of the manuscript.
COMPETING INTERESTS

The authors have no competing interests.

DATA SHARING

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REFERENCES


23. Wells JM, Jackson PL, O'Reilly PJ, Blalock JE. Selective Inhibition Of Leukotriene A4 Hydrolase Aminopeptidase Activity Occurs In COPD And Reflects Clinical Outcomes. American journal of respiratory and critical care medicine 2012;185:A1422
FIGURE LEGENDS

Figure 1. **Azithromycin treatment decreases PGP levels in sputum of COPD patients** compared with placebo. Inset: values represent the mean ± SEM PGP concentrations for sputa (*p<0.05 vs. placebo).

Figure 2. **Sputum PGP levels decline with increased duration of azithromycin therapy.** PGP levels were significantly higher in subjects treated with placebo compared to azithromycin at months nine and twelve of therapy (*p<0.02) but not at months one through six (p=0.32).

Figure 3. **Sputum PGP levels are highest around the time of a COPD exacerbation.** Values represent PGP levels at various days relative to the onset of an exacerbation (time 0). Inset: values represent the mean ± SEM PGP concentrations for sputa collected 103 ± 37 days before an exacerbation (pre), 18 ± 4 days before or after an exacerbation (peri) or 141 ± 11 days after an exacerbation (post). *p<0.02 (pre vs. peri), #p<0.02 (post vs. peri)
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