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Saunders, R., Kaul, H., Berair, R. et al. (2019) DP2 antagonism reduces airway smooth muscle mass in asthma by decreasing eosinophilia and myofibroblast recruitment. Science Translational Medicine, 11 (479). ISSN: 1946-6234

https://doi.org/10.1126/scitranslmed.aao6451

DP_2 antagonism reduces airway smooth muscle mass in asthma by decreasing eosinophilia and myofibroblast recruitment

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Overline: Asthma

One sentence summary: Cellular and computational models and bronchial biopsies from asthma patients show that a DP_2 antagonist reduces airway smooth muscle mass in asthma.

Abstract

Increased airway smooth muscle mass, a feature of airway remodeling in asthma, is the strongest predictor of airflow limitation and contributes to asthma-associated morbidity and mortality. No current drug therapy for asthma is known to affect airway smooth muscle mass. Although there is increasing evidence that prostaglandin D_2 type 2 receptor (DP_2) is expressed in airway structural and inflammatory cells, few studies have addressed the expression and function of DP_2 in airway smooth muscle cells. We report that the DP_2 antagonist fevipiprant reduced airway smooth muscle mass in bronchial biopsies from patients with asthma who had participated in a previous randomized placebo-controlled trial. We developed a computational model to capture airway remodeling. Our model predicted that a reduction in airway eosinophilia alone was insufficient to explain the clinically observed decrease in airway smooth muscle mass without a concomitant reduction in the recruitment of airway smooth muscle cells or their precursors to airway smooth muscle bundles that comprise the airway smooth muscle layer. We experimentally confirmed that airway smooth muscle migration could be inhibited in vitro using DP_2-specific antagonists in an airway smooth muscle cell culture model. Our analyses suggest that fevipiprant, through antagonism of DP_2, reduced airway smooth muscle mass in patients with asthma by decreasing airway eosinophilia in concert with reduced recruitment of myofibroblasts and fibrocytes to the airway smooth muscle bundle. Fevipiprant may thus represent a potential therapy to ameliorate airway remodeling in asthma.

Introduction

Asthma affects over 300 million people worldwide and its prevalence is increasing (1) despite currently available therapies (2). Asthma is characterised by variable airflow limitation that becomes more persistent in severe disease. Increased airway smooth muscle (ASM) mass is an important component of airway remodeling in asthma, contributing substantially to symptoms and disordered airway physiology (3-5). To date, no drug has impacted upon the increased ASM mass observed in asthma in randomized placebo-controlled trials (3, 4). However, bronchial thermoplasty has demonstrated a potential reduction in ASM mass in asthma in uncontrolled studies (6-7).

The prostaglandin (PG) D_2 type 2 receptor (DP_2, also known as chemoattractant receptor-homologous molecule expressed on T helper [Th] 2 cells [CRTh2]) is expressed by inflammatory cells critical in the immunopathogenesis of asthma, including eosinophils, Th2 lymphocytes, type 2 innate lymphoid cells, and mast cells. DP_2 activation promotes cellular release of cytokines, inflammatory cell migration, and cell survival (8-10). Its archetypal ligand PGD_2 is predominantly released by mast cells localized to the ASM-bundle (11). The DP_2 antagonist fevipiprant has been shown to improve asthma symptoms, lung function, airway eosinophilia, and epithelial integrity (4). However, the role of the PGD_2/DP_2 axis in ASM dysfunction in asthma has not been extensively studied. We hypothesized that the PGD_2/DP_2 axis may contribute to increased ASM mass in asthma, and that antagonism of DP_2 with fevipiprant might result in a decrease in ASM mass.

Here, we analysed bronchial biopsies from asthma patients treated with the DP_2 antagonist fevipiprant in a previous phase 2a randomized, placebo-controlled trial undertaken to determine the impact of drug upon airway inflammation, remodeling and asthma control (4).

Using an agent-based computational model representing an asthmatic airway in human patients and supported by in vitro ASM cell-based observations, we propose that the reduced ASM mass observed in the bronchial biopsies after fevipiprant treatment may be a consequence of inhibition of eosinophilic airway inflammation together with reduced recruitment of myofibroblasts and fibrocytes to the ASM bundle.

Results

Fevipiprant reduces ASM mass in subjects with asthma in a randomized placebo-controlled trial

We obtained bronchial biopsies from moderate-to-severe asthmatics with airway eosinophilic inflammation, as evidenced by increased sputum eosinophil counts. These individuals had participated in a 12-week single-centre (University of Leicester), randomized, double-blind, parallel-group, placebo-controlled trial of the DP_2 antagonist fevipiprant (4). We then performed an a priori quantification of ASM mass (percentage of total bronchial biopsy area) (11). A representative photomicrograph of a bronchial biopsy from a subject showing disrupted epithelium and increased ASM mass is shown in Fig. 1A. The absolute ASM mass percentage (mean ± standard error of the mean [SEM]) observed decreased significantly following treatment with fevipiprant (-13 ± 5%; p=0.022, n=14) versus placebo (4 ± 5%; p=0.52, n=13) (mean difference [95% confidence interval (CI)] -16.2 [-1.4 to -31.1] %; p=0.034) (Fig. 1B). In view of the above data, we performed a post hoc quantification of ASM mass (percentage of total biopsy area) in bronchial biopsies derived from a sub-group of moderate-to-severe eosinophilic asthmatics who had participated in a 50-week single-centre (University of Leicester), randomized, double-blind, parallel-group, placebo-controlled trial of the anti-interleukin (IL)-5 neutralizing antibody mepolizumab (12). Although the sample size was small, in contrast to fevipiprant, we observed no significant effect of mepolizumab on

ASM mass (absolute ASM mass percentage increase post-mepolizumab 2.9 ± 4.0%, n=7 versus placebo 1.5 ± 2.2%, n=5) (mean difference [95% CI] 1.4 [-9.9 to 12.7] %; p=0.79). For these and other data, see Data File S1.

An agent-based computational model recapitulates the features of airway remodeling observed in asthma

To interrogate the mechanisms governing the pathogenesis of asthma, we developed an agent-based computational model of airway remodeling comprising epithelial, mesenchymal, and inflammatory parameters. In agent-based modeling, a system is divided into agents (here, airway cells; table S1) capable of interacting with each other and their environment based on defined rule-sets (13-15). The initial state of the model is illustrated in Fig. S1. Our model considered interactions between epithelial (columnar and goblet cells), mesenchymal (fibroblast, myofibroblast, and ASM cells), and inflammatory (eosinophil) cell types. The various cell types, depending on their phenotype, displayed behaviours ranging from proliferation, migration, (de)differentiation, apoptosis, and synthesis of extracellular matrix (ECM) proteins and cytokines (table S1). These virtual cells were simulated within a Strahler Order 3 virtual airway with a lumen diameter of 1.21 mm and wall area of 1.79 mm^2. The number of each cell type within the model was based on geometrical constraints and published data. The rule-set governing agent behaviours and interactions was derived from existing in vitro, animal, and clinical studies (table 1). The underpinning agent interactions and rule-sets attributed to the agents are summarized in table 1 and represented schematically in Fig. S2.

In the model, we initially damaged the epithelium to cause 50% epithelial denudation. We then simulated the consequent normal injury repair and pathological airway remodeling over 180 days by introducing alterations to model parameters (tables S2 – S4). The following

pathological markers and value ranges were considered necessary in the model to reflect the key hallmarks of severe asthma (1, 2): eosinophilic inflammation (eosinophils/mm^2 sub-mucosa) >10; epithelial integrity <70%; and ASM mass ≥10% and ≤50%. We conducted parametric testing by varying, both individually and collectively, parameters from all agent categories and observing which conditions best captured the above hallmarks of asthma. We defined the most parsimonious set of parameters to capture these three hallmarks (tables S2 – S4). The response to epithelial injury in this model displayed significantly increased eosinophilic inflammation ($p<0.001$), ASM mass ($p=0.002$) and persistent epithelial damage ($p<0.001$) compared to the model of healthy control individuals over the 180-day time course (Fig. 2A).

Computational modeling predicts that a reduction in eosinophil recruitment is not sufficient to decrease ASM mass

In order to predict the impact of reducing eosinophil number in our model of airway remodeling, we incorporated pro-apoptotic or anti-recruitment elements into the model. These variables were chosen to represent the major respective effects of neutralizing IL-5 (12, 26), an obligate cytokine for eosinophil survival and maturation, and of blocking activation of DP_2, which promotes eosinophil recruitment (4). We tested an increasing range of intervention doses and found they resulted in a progressive reduction in airway eosinophilia and ASM mass in our model of airway remodeling (Fig. 2B, C).

We then used our computational model of a remodeled asthmatic airway to determine the predicted percentage increase in eosinophil apoptosis and percentage reduction in eosinophil recruitment required to reduce the number of bronchial wall eosinophils to that seen in vivo in clinical trials of mepolizumab (27) and fevipiprant (4), which reduced the eosinophil count in

patients by 55% and 80%, respectively compared to placebo control. To attain the reduction in airway eosinophil number clinically observed with mepolizumab, the model predicted that 15% of the eosinophil population must be induced to undergo apoptosis (reduction in airway eosinophilia versus control of 54.1 ± 4.1 %*;* Fig. 2B, chequered bar). To attain the reduction in airway eosinophil number clinically observed for fevipiprant, the model predicted that a 40% reduction in eosinophil recruitment was required (reduction in airway eosinophilia versus control of 81 ± 0.6 %; Fig. 2B, hatched bar).

We subsequently used the pro-apoptotic (15%) and anti-recruitment (40%) models resulting in a reduction in eosinophil number equivalent to that seen in the mepolizumab and fevipiprant clinical trials to predict the impact of each intervention on ASM mass. When assuming an increase in eosinophil apoptosis of 15%, the pro-apoptosis model predicted a small mean ± SEM decrease in ASM mass (absolute reduction 4.0 ± 0.6%; relative reduction 12 ± 2% versus control; Fig. 2C, chequered bar). This is consistent with the mepolizumab clinical trial (12) in which no significant change in ASM mass was observed. When assuming a decrease in eosinophil recruitment of 40%, the anti-recruitment model predicted a modest reduction in ASM mass (absolute reduction 8.1 ± 0.5%; relative reduction 25 ± 1% versus control; Fig. 2C, hatched bar), which was not sufficient to result in the observed response to fevipiprant (13% absolute and 44% relative reduction in ASM mass). The model therefore suggested the existence of additional mechanisms that, along with a reduction in airway eosinophilia, mediated the reduction in ASM mass following treatment with fevipiprant.

The ASM PGD_2/DP_2 axis mediates ASM migration

To explore the mechanism by which DP_2 antagonism resulted in a decrease in ASM mass, we assessed the expression and function of DP_2 in ASM. We found that DP_2 was expressed in the

ASM-bundle in bronchial biopsies from patients recruited for research bronchoscopies (Fig. 3A), in line with the previous finding that PGD_2 primes migration of ASM cells towards platelet-derived growth factor via DP_2 (28). However, DP_2 expression was not significantly different between subjects with severe asthma (60 ± 1; n=8) and healthy controls (57 ± 5; n=11) (mean difference [95% CI] 2.6 [-9.9 to 15.0]; p=0.67). We also confirmed DP_2 expression in primary human ASM cells at the mRNA (Fig. 3B*)* and protein levels (Fig. 3 C, D and Fig. S3).

PGD_2 can activate PGD_2 type 1 (DP_1), DP_2 and thromboxane (TP) receptors (9). Therefore, we investigated the effect of the selective DP_2 agonist 13,14-Dihydro-15-keto-PGD_2 (DK-PGD_2) and selective DP_2 antagonists (fevipiprant, CAY10471 and OC000459) on DP_2 receptor activation, phenotype and behaviour of primary human ASM cells. DK-PGD_2 (10-100nM) stimulated a small but significant increase in filamentous actin (F-actin) polymerisation and intracellular calcium elevation (Fig. 3 E-F, area under curve of the dose response [AUC DR] p=0.01 and p=0.002, respectively). Although this did not translate to an effect of DK-PGD_2 on ASM cell migration in vitro (Fig. 4A), blocking activation of DP_2 by endogenous PGD_2 with the DP_2 antagonist fevipiprant significantly inhibited ASM cell migration in vitro at the highest drug concentrations (percentage reduction in cells migrating into the wound after 24h vs vehicle control: 10 nM, 8.8 ± 7.8, p=0.294; 50 nM, 7.7 ± 7.4, p=0.332; 100 nM, 12.8 ± 4.9, p=0.034, 500 nM, 17.4 ± 6.6, p=0.034; Fig. 4A). We confirmed this effect using other DP_2 antagonists including CAY10471 (percentage reduction in cells migrating into the wound after 24h vs vehicle control: 10 nM, 11.6 ± 2.2, p=0.010; 50 nM, 9.3 ± 3.7, p=0.038; 100 nM, 13.6 ± 4.4, p=0.027; Fig. 4A), and OC000459 (percentage reduction in cells migrating into the wound after 24h vs vehicle control: 10 nM, 8.4 ± 3.3, p=0.043; 50 nM, 6.7 ± 4.2, p=0.157; 100 nM, 8.8 ± 1.9, p=0.003, Fig. 4A). Representative photomicrographs of primary ASM monolayer cultures wounded by scratching followed by incubation with different treatments

for 24h are shown in Fig. 4B. Thus, we hypothesized that PGD_2 was released into the extracellular milieu by ASM cells to affect ASM behaviour in an autocrine manner. Indeed, genes involved in PGD_2 biosynthesis and metabolism, including PGD_2 synthase, were expressed by ASM cells from subjects with and without asthma (tables S5 and S6). Consistent with previous reports (29), PGD_2 was released by ASM, albeit at a low concentration compared with mast cells (30-31), and this PGD_2 release increased following wounding (129 ± 18 versus 176 ± 22 pg PGD_2/ml/10^5 ASM, mean difference [95% CI] 52.0 [4.8 to 99.2]; p=0.02, Fig. 4C). In addition to myofibroblasts, we demonstrated that the ASM progenitor fibrocytes expressed DP_2 (Fig. 4D). The correlation between the change in ASM percentage observed in those treated with fevipiprant or placebo and the change in lamina propria myofibroblast or fibrocyte number supported the view that the effects of fevipiprant on ASM mass and lamina propria mesenchymal cells may have occurred in parallel (Fig. 4E, F). These findings suggested that anti-DP_2 might, in part, reduce ASM mass via a direct and concomitant effect upon ASM and myofibroblast or fibrocyte recruitment to the ASM bundle.

The effects on ASM cell migration were not due to cytotoxic effects on the ASM cells as there was no effect on cell number following treatment for 24h with DK-PGD_2 (100 nM), fevipiprant (500 nM), CAY10471 (100 nM) or OC000459 (100 nM) (Fig. S4A). DK-PGD_2 (100 nM), fevipiprant (500 nM) and CAY10471 (100 nM) had no effect on apoptosis or necrosis (Fig. S4B). This was supported by a lack of effect of DK-PGD_2 (100 nM), fevipiprant (500 nM) or CAY10471 (100 nM) on cell size or granularity, which are known to change during apoptosis/necrosis (Fig. S4C-D). Furthermore, DK-PGD_2 (10 - 100 nM, Fig. S5A), CAY10471 (10 - 100 nM, Fig. S5B) and fevipiprant (10 - 500 nM, Fig. S5C) neither induced proliferation of ASM in the presence of serum-free media nor inhibited the ASM cell proliferation induced by fetal bovine serum (FBS) over 3 days, as assessed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-

carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium inner salt (MTS) assay and carboxyfluorescein succinimidyl ester (CFSE) fluorescence (Fig. S5D, E). In the fevipiprant clinical trial, there was no change in proliferating cell nuclear antigen (PCNA) staining in bronchial biopsies pre- versus post- fevipiprant treatment or placebo after 12 weeks of treatment. Additionally, DK-PGD_2 (10 – 100 nM), fevipiprant (10-500 nM) or CAY10471 (10-100nM) had no effect on ASM α-smooth muscle actin (SMA) expression (Fig. S6A). This was supported by a lack of effect of DK-PGD_2 (100 nM), fevipiprant (500 nM) or CAY10471 (100 nM) on basal or bradykinin (BK)-stimulated ASM contraction (Fig. S6B-C).

Modeling predicts that reductions in myofibroblast and eosinophil recruitment are required for fevipiprant to decrease ASM mass

To support our in vitro findings, we reduced the myofibroblast recruitment (0-50%) in the computational model together with the 40% reduction in eosinophil recruitment required to reflect the observed reduction in bronchial biopsy eosinophils after fevipiprant treatment in patients with asthma as described above. The resulting model predicted that a 50% reduction in myofibroblast recruitment in concert with reduced eosinophil recruitment would result in a decrease in ASM mass equivalent to that seen after fevipiprant treatment (cross hatched bars, Fig. 4G). In contrast, a reduction in myofibroblast recruitment alone was predicted to result in minimal effects on ASM mass (1.8 ± 1.2% relative reduction). A comparison of the computational model and fevipiprant trial findings is summarised in Fig. S7.

Discussion

We report that a drug intervention in asthma, namely fevipiprant (a DP_2 antagonist), reduced ASM mass in bronchial biopsies from asthma patients who had participated in a previous randomized placebo-controlled trial (4). This is in contrast to the lack of an effect on ASM

mass in response to mepolizumab that we report here, corticosteroids or the anti-IL-13 monoconal antibody tralokinumab (3, 32, 33). Our computational model and in vitro work supported the view that the reduction in ASM mass in response to fevipiprant was a consequence of inhibiting eosinophilic inflammation in concert with a direct reduction in the recruitment of myofibroblasts to the ASM-bundle. Thus, fevipiprant may be a potential therapy to target airway remodeling in asthma and its clinical benefits observed previously could be in part due to its effects upon ASM**.**

One limitation of our study is that the number of paired biopsies collected in the fevipiprant trial included modest numbers of subjects despite the trial being one of the largest biopsy studies undertaken in subjects with asthma. Therefore, it is important to extend and confirm these findings in future studies. Likewise, it is possible that the lack of effect observed with other anti-inflammatory interventions is due to lack of statistical power conferred by the small sample sizes. However, our computational model data suggest that these anti-inflammatory approaches are unlikely to be effective unless they have additional direct effects upon ASM. Indeed, a small reduction in ASM mass was previously reported following treatment with the calcium channel blocker gallopamil, which had been proposed to have direct effects upon ASM activation, but the reduction in ASM mass was no different from placebo (34).

Another limitation of our study is that we cannot completely exclude the possibility that the effect of fevipiprant upon ASM mass both in vivo and in vitro is an off-target effect. However, we used 3 selective and specific DP_2 antagonists, including fevipiprant, for the in vitro experiments, and therefore we consider it unlikely that the findings we report on ASM activation and migration are due to off-target effects. Our in vitro findings also imply that the major effect of DP_2 antagonism upon ASM function was the inhibition of migration of ASM

progenitors to the airway, either from the blood or via attenuation of epithelial-mesenchymal transition, rather than through effects on proliferation or apoptosis. This is consistent with the concept that mesenchymal cells exhibit plasticity in phenotype (35). In keeping with our in vitro observations, we did not identify any changes in PCNA staining in the ASM bundle in vivo, suggesting there was no active proliferation of ASM. Taken together, these results suggest that neither proliferation nor apoptosis contribute to the effects of DP_2 antagonism upon ASM mass, although we cannot completely exclude some contribution from these processes.

A strength of our study is the integration of findings from in vivo clinical trials and in vitro and computational models. We developed a comprehensive agent-based model of airway remodeling during asthma. Previous computational approaches have been applied to uncover mechanisms driving unresolved allergic inflammation and airway hyper-responsiveness in asthma (15, 36), but not airway remodeling. Our agent-based model was created to represent the airway in 3-dimensions. A possible limitation is that our model was utilized for one layer of agents and simulated a distal airway to balance model resolution and computational complexity. However, we believe our model is representative as it captures the features of the normal and pathological workings of the entire airway. Specifically, our computational model displayed features consistent with moderate-to-severe asthma including damaged bronchial epithelium, eosinophilic inflammation and increased ASM mass. This model responded to perturbations reflective of changes in eosinophil survival and trafficking and provided new insights into possible mechanisms of action of DP_2 antagonists versus anti-IL5 upon airway remodeling. This model has also given us insights into the effects of DP_2 antagonism, which would not be possible in vitro due to the limitations of studying multiple cell-cell interactions within a complex airway structure. Although our computational model is not patient-specific

295 it represents an average patient with asthma and airway remodeling. This ‘virtual patient’
296 represents a step towards patient-specific modeling in respiratory medicine. We anticipate our
297 integrated approach combining agent-based modeling with in vivo clinical data and in vitro
298 findings will provide further insights into asthma in future studies.

Materials and methods

Study design

The objective of the study was to use an integrated strategy encompassing samples from a randomized placebo-controlled trial in asthma patients evaluating fevipiprant (DP_2 antagonist) and mepolizumab (anti-interleukin-5 antibody), in vitro experiments, and predictive computational agent-based models simulating asthma pathogenesis to investigate the impact of DP_2 antagonism upon ASM mass and determine the mechanisms driving this effect.

Subjects with persistent moderate-to-severe asthma and an elevated sputum eosinophil count (n=61) participated in a single-centre (University of Leicester) randomized placebo-controlled trial of the DP_2 antagonist fevipiprant (225mg twice per day orally) in addition to standard of care (4). In an independent study, subjects (n=61) who had refractory eosinophilic asthma participated in a single-centre (University of Leicester), randomized placebo-controlled trial of an anti-interleukin-5 neutralising antibody mepolizumab (750 mg intravenous infusions every 4 weeks over 50 weeks) in addition to standard of care (12). A subgroup of subjects underwent bronchoscopy and bronchial biopsy in each independent study before and after administration of drug or placebo. The studies were approved by the Leicester and Northamptonshire ethics committee (05/Q2502/98 and 11/EM/0402, respectively) and registered with ClinicalTrials.gov (ISRCTN75169762, NCT01545726 and with EudraCT, number 2011-004966-13). The studies were carried out in accordance with CONSORT guidelines (4, 12).

The sample size for the fevipiprant and mepolizumab randomized controlled trials were determined based on change in the sputum eosinophil count as the primary outcome as described in Gonem et al (4) and on the number of exacerbations of asthma per subject as the primary outcome as described in Haldar et al (12). Assessing change in airway smooth muscle

mass was in the pre-specified exploratory analysis plan for the fevipiprant clinical trial, and was performed post-hoc for mepolizumab. The inclusion and exclusion criteria for the fevipiprant and mepolizumab trials, randomisation and blinding procedures are described in Gonem et al (4) and Haldar et al (12), respectively. For the in vitro experiments the observers analysed the experiment blinded to conditions.

Additional asthmatic subjects and healthy controls were recruited from a single-centre (University of Leicester) for research bronchoscopies from which tissue sections and primary ASM cells could be derived. Those with asthma gave an appropriate history and had objective evidence of variable airflow obstruction or airway hyper-responsiveness, as described previously (37). Healthy controls had no history of asthma and possessed normal lung function. The study was approved by the Leicestershire and Northamptonshire Ethics Committee (08/H0406/189).

Immunohistochemistry

To determine DP_2 expression by ASM, bronchial biopsies from healthy controls (n=11 donors) and asthmatic subjects (n=8 donors) were embedded in glycomethacrylate (GMA) (11). For each subject, sequential 2 μm sections were cut and stained using polyclonal anti-DP_2 antibody (Thermo-Fisher Scientific) or rabbit immunoglobulin (Ig) G isotype control (Immunostep), and an α-SMA antibody (clone 1A4, Dako) or mouse IgG2a isotype control (clone DAK-GO5, Dako). Antibody binding was detected using the EnVision FLEX kit (Dako). For determining ASM mass pre- and post-treatment with fevipiprant (n=14) or placebo (n=13), and pre- and post-treatment with mepolizumab (n=7) or placebo (n=5) bronchial biopsies from asthmatic subjects were embedded in GMA and stained for α-SMA as above. ASM mass was determined as the percentage of the total assessable biopsy area as previously described by a single

observer (RB). Repeatability of ASM mass assessment was tested and was excellent within and between observers with intraclass correlations of 0.95 and 0.96 respectively. Myofibroblasts were identified as α-SMA positive stained cells in the lamina propria that were neither located as part of the ASM-bundle nor as vascular smooth muscle cells adjacent to vessels per mm^2 of submucosa. To identify fibrocytes in bronchial biopsies pre- and post-fevipiprant (n=12) or placebo (n=13), for each subject sequential 2 μm sections were cut and stained using an mouse monoclonal anti-cluster of differentiation (CD) 34 antibody (Dako) or mouse IgG1 isotype control (Dako) and α-SMA as above. Fibrocytes were identified as the subset of α-SMA positive cells/mm2 lamina propria that also stained positive for CD34 in sequential sections. The intensity of DP_2 stain was quantified as reciprocal intensity (38) on a scale out of 250, assessed by a single observer. Assessors were blind to clinical characteristics, treatment allocation and order of bronchial biopsy in the clinical trial.

Cell culture

ASM bundles were isolated from bronchial biopsies (n=27 asthmatic, 2 non-asthmatic) and lung resection material (n=4, non-asthmatic). The clinical characteristics of subjects that underwent bronchoscopy to provide primary ASM cultures are as shown (table S5). Primary ASM cells were cultured in DMEM with Glutamax-1 supplemented with 10% FBS, 100U/mL penicillin, 100μg/mL streptomycin, 0.25 μg/mL amphotericin, 100 μM non-essential amino-acids, and 1 mM sodium pyruvate (Gibco). Cells were characterized for α-SMA expression using a mouse monoclonal anti-α-SMA antibody (clone 1A4, Dako) or mouse IgG2a isotype control (clone DAK-GO5, Dako) by flow cytometry and used between passage 2-6.

Fibrocytes (n=6) were isolated from peripheral blood mononuclear cells as described previously (39). PBMCs were washed twice with HBSS and cultured in tissue culture flasks coated with 40 μg/ml fibronectin for 5 -10 days prior to experimentation.

Prior to experimentation ASM cells from each individual donor were incubated in media in the presence of a selective DP_2 agonist (DK-PGD_2, Cayman Chemical Company (40)) or selective DP_2 antagonists (CAY10471, OC000459 and fevipiprant, Cayman Chemical Company and Novartis (41-43) vs appropriate vehicle controls (Dimethyl sulfoxide (DMSO) for DK-PGD_2, CAY10471 and OC000459, and 10% dH20 in DMSO for fevipiprant).

Wound healing assay

ASM cells from individual donors were seeded onto 6 well plates coated with 10 μg/ml fibronectin at a density of 2×10^5 cells and allowed to adhere and reach 90-100% confluence. Cells were then serum deprived for 24h. Cells were wounded by scratching using a sterile 200 μl pipette tip in a predetermined grid pattern (44). Following wounding ASM cells were washed x4 with serum free media prior to addition of serum free media with DK-PGD_2 (10-100 nM), fevipiprant (10-500 nM), CAY10471 (10-100 nM) or OC000459 (10-100 nM) or vehicle control for 24 h. Photographs of 4 different wounded areas per condition were then photographed at baseline and after 24h using an EVOS xl core cell imaging system (Thermo Fisher Scientific), and the outline of the wound at time zero transposed onto the corresponding 24h photograph. The number of cells that had moved into the wounds were analysed by a blinded observer.

Computational model approach and framework

The computational model capturing airway remodeling was developed via the agent-oriented approach (13), which charts the spatiotemporal evolution of a system as a result of flexible, high-level interactions between agents as well as agents and their environment (14). The *Flexible Large-scale Agent-based Modelling Environment* (www.flame.ac.uk), an agent-based platform employing *stream communicating X-machines* (45) as agents, was utilized to develop the model.

The baseline model and computational iterations

Based on the agents, set of rules, and initial conditions (table 1, table S1, Fig. S1, Fig. S2, supplementary materials) a baseline model of airway remodeling, comprising epithelial, mesenchymal, and inflammatory parameters, was developed that captures trends observed during normal airway remodeling, and additionally following introduction of abnormal levels of variation within model parameters leads to the emergence of patterns observed during pathological remodeling and, as such, the key hallmarks of asthma – this approach is referred to as *pattern-oriented modelling* (13). After review of clinical literature, the following clinical markers were considered to appropriately reflect the key hallmarks of asthma: i) eosinophilic inflammation (defined as eosinophils/mm^2 submucosal area) >10 (5, 11, 46), ii) epithelial integrity <70% (4-5, 47-50) and iii) airway muscle mass/wall area ≥10% and ≤50% (4-5, 47, 49).

The model starts by assessing the epithelial integrity, the number and location of inflammatory cells (both the universal inflammatory cells and eosinophils), and the status of muscle cells. Remodeling is initiated in case of a compromised epithelium or increased inflammation within the system, resulting in a cascade of events, which, depending on the relevant boundary

conditions, lead to further inflammation, fibrosis, goblet cell hyperplasia, recruitment of muscle, and increased collagen deposition. Furthermore, remodeling, could be exacerbated or prolonged by the nature of initial or secondary conditions assigned to the computation.

More specifically, a normal or 'healthy' set of conditions triggered remodeling in the absence of an intact epithelium (or a challenge that resulted in epithelial denudation) by initiating fibrosis and recruiting the universal inflammatory cell. The inflammatory cell further 'released' pro-inflammatory cytokines to recruit eosinophils and muscle – the latter was accounted for in the model by the differentiation of fibroblasts into myofibroblasts. The eosinophils, moreover, caused further damage by degranulating and releasing cytotoxic proteins, which, if close to the epithelium, resulted in necrosis of the epithelial cells – thereby, prolonging remodeling. We worked with the hypothesis that any set of conditions that perpetuate these interactions will result in pathological remodeling, thereby capturing the hallmarks of asthma. The various parameters and their quantitative values, derived from existing literature, have been, along with the relevant references, listed in table 1, with a schematic interlinking the various elements of the model shown in Fig. S2.

While the model does not explicitly consider cytokine activity, i.e. their release, diffusion, and half-life, it implicitly accounts for it by requiring that those cells impacted by the cytokine molecules share a localized region with the effector cell. For example, only ASM cells within close proximity of the universal inflammatory cellswill undergo hypertrophy or contraction, refer to supplementary material for more details.

Finally, the simulations progress in a number of time steps, with each time step matching 1 hour of real time. Time intervals of 30 minutes and 2 hours were also tested on the baseline

445 case (Case I) and yielded results indistinguishable from simulations conducted with 1-hour
446 time steps. Thus, we opted for the 1-hour interval to strike a balance between computational
447 costs and ensuring adequate resolution regarding activities we wished to capture via the model.
448 The total simulated time for all simulations, including remodeling and intervention, was ~6
449 months (4350 iterations). This time period allowed investigation of both the short- and long-
450 term response following either a challenge or intervention. Each model was simulated five
451 times (n=5) to assess the sensitivity of the model to inherent stochastic elements (such as cell
452 cycle, new coordinates of the daughter cells and migration of the universal inflammatory cells).
453 The internal random elements accounted for intra- and inter-cellular biological stochasticity.
454 Testing model for insensitivity to these random elements also served to provide an indicator
455 for model precision.
456
457 A detailed description of the model, its development, and its validation is provided in the
458 supplementary materials.
459
460 **Statistical analysis**
461 Statistical analysis was performed using with SAS/STAT software and GraphPad Prism. Data
462 were tested for normality using the Shapiro-Wilk test. For normally distributed data, two-tailed
463 paired t-tests, one sample t-tests, or one-way ANOVA were used as appropriate. For non-
464 parametric data, Wilcoxon matched pairs sign ranked test or Kruskal-Wallis tests were used as
465 appropriate. Correlations were performed using Spearman's correlation. Details of statistical
466 tests used are provided in figure legends. $p < 0.05$ was considered statistically significant.

List of Supplementary Materials

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Acknowledgements

We thank Marie Laurencin, Ruth Hartley, Vijay Mistry, Adel Mansur for their contribution to the fevipiprant clinical trial, all the clinical staff involved in collecting samples and patient details, the patients who participated in this study, Natasha Johnson for technical assistance, Fay Hollins for assistance with illustrations and Igor Chernyavsky for critical review of the computational modeling.

Funding

This research was co-funded by the National Institute for Health Research (NIHR) Leicester Biomedical Research Centre (CEB, AJW, SS), the National Institute for Health Research (NIHR) Oxford Biomedical Research Centre (IDP), Novartis (to CEB, SS), Airway Disease Predicting Outcomes through Patient Specific Computational Modelling (AirPROM) project (funded through 7th EU framework grant, 270194, to CEB, SS, BSB, RHS), and Wellcome Trust Senior Fellowship (WT082265, to CEB). This paper presents independent research funded by the National Institute for Health Research (NIHR). The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

Author contributions

All authors contributed to the study concept and overall study design and read, edited and approved the final manuscript. RS designed and conducted the in vitro experiments, analysed data and wrote the draft manuscript. LC, RB, DK, AJS and MSB contributed to the design and undertaking of experiments, analysed data and contributed to figures. HK participated in computational modeling study design, developed the various agent-based models, conducted the computational modeling, analysed the data and wrote the draft manuscript. RHS, CEB, SS and BSB helped conceive the computational modeling. CEB, IDP, SS, AJW, RAK, RB, SG,

AS and MB contributed to the design and delivery of the clinical trials, recruitment and characterisation of patients. CEB conceived the study, participated in experimental design and wrote the draft manuscript.

Conflicts of interest

CEB serves on advisory boards for GlaxoSmithKline, AstraZeneca, Boehringer Ingelheim, Cheisi, Roche, receives honoraria from Novartis, and receives research support from GlaxoSmithKline, AstraZeneca, Chiesi, Novartis, Boehringer Ingelheim and Roche. AJW serves on Advisory Boards for GSK, Astra Zeneca, Pulmocide, KNOPP Pharmaceuticals and Anaxsys. In the last 5 years IDP has received speaker's honoraria for speaking at sponsored meetings from Astra Zeneca, Boehringer Inglehiem, Aerocrine, Almirall, Novartis, Teva and GSK and a payment for organising an educational event from AZ. IDP has received honoraria for attending advisory panels with Almirall, Genentech, Regeneron, Astra Zeneca, Boehringer Ingelheim, GSK, MSD, Schering-Plough, Novartis, Dey, Napp, Teva, Merck and Respivert. IDP has received sponsorship to attend international scientific meetings from Boehringer Ingelheim, GSK, Astra Zeneca, Teva and Napp. SG has received support to attend scientific conferences from GSK and Chiesi. At the time of this study RAK was an employee of Novartis Pharmaceuticals AG. SS has performed advisory services for Mundipharma, GSK, Astra Zeneca, Roche, Boehringer Ingelheim & Owlstone Medical.

Data and materials availability

The agent-based model is available from figshare, doi: 10.25392/leicester.data.7610933 for research purposes under the Creative Commons Attribution Non-Commercial 4.0 International (CC BY-NC 4.0) license. Fevipiprant is in phase 3 trials and until licensed is not available to other researchers to undertake clinical trials without permission from Novartis. Fevipiprant was

755 provided to the University of Leicester under a Material Transfer Agreement and is also
756 available from commercial suppliers. The gene array data have been deposited in the
757 ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number
758 E-MTAB-7346. All data are present in the main text or in the Supplementary Materials.

Category	Activity	Rules	Parameters	Baseline Value	Comments
Epithelial	Migration	Dedifferentiated epithelial cells migrate to repair the damaged epithelium, then undergo proliferation.	Migration rate	5 μm/h	Migration rate was consistent with in vitro studies (16-18).
			Migration delay	5h	Dedifferentiated cells migrate after 5h; doubled to 10 h to represent slowly recovering epithelium.
	Differentiation	Cells at the edge dedifferentiate into a flattened phenotype.	Dedifferentiation probability	50%	A 50% probability per iteration was applied to determine whether an epithelial cell dedifferentiates. This was altered to 5% per iteration for the defective epithelium.
			Delay in proliferation	2-8 h	Edge cells require 2-8h to flatten out (16); this was increased by 3X (6–24h) for slow epithelial recovery.
	Proliferation	Dedifferentiated epithelial cells proliferate following their migration to the opposite edge of a compromised epithelium.	Ciliated:**Goblet** ratio	0.7:**0.3** & 0.1:**0.9**	The number of goblet cells increase by 2–3X in severe asthma (19-22) given the inflammation status in disease. The probability of differentiating to a ciliated: goblet cell fate was either 0.7:**0.3** (less inflamed) or 0.1:**0.9** (more inflamed).
	Apoptosis	This feature was **not** included in the baseline model.	Auto-denudation	N/A	No apoptosis rate was applied to the baseline model. In 'mild' shedding every 25 h cells underwent apoptosis with 50% probability; 'moderate' apoptosis with the probability of 1% at ***each*** iteration; 'extreme' 10% at ***each*** iteration.
Inflammatory	Cell migration and activation	Inflammatory cells migrate into the airway in response to epithelial denudation, and their activation promotes eosinophil recruitment.	Frequency of recruitment	120 h	Inflammatory cells were added every 120h to the model with recruitment frequency increased to 60h and 50h in disease.
			Inflammatory cells recruited	30	Inflammatory cell number was chosen to reflect previous reports (11) and, in disease, this was increased by 1.5-2X (23).
			Eosinophil recruitment	45	Eosinophil recruitment was triggered by increased inflammatory cell number; 45, based on previous reports (11) and in disease this threshold was halved.
	Degranulation	Inflammatory cell activation promotes cell recruitment and fibroblast differentiation	Inflammatory cytokine release	25/ 75/ 105 h	The 'universal' inflammatory cell was assigned 150h life; releasing cytokines for 25h (or 75 and 105h for epithelial integrity of <55% and 30% respectively). In disease, inflammatory cell life was reduced to 65, 40, and 25 h (releasing cytokines for 85, 110, and 125h, respectively).
	Apoptosis	Inflammatory cells promote eosinophil survival	Eosinophil life in the airway	10/ 30/ 45h	Eosinophils survival is ~10h within a normal airway (24). This was increased to 30 and 45h for epithelial integrity of <55% and 30% respectively. In disease, the life of eosinophils was doubled to 20, 60, and 90 h.
Mesenchymal	Differentiation	Inflammatory cells promote increased ASM mass	ASM activation	25 cells	The threshold number of inflammatory cells above which myofibroblast-ASM differentiation is induced (11) which was reduced by 50% in disease.
	Differentiation	Inflammatory cells promote fibroblast-myofibroblast differentiation	(Myo)fibroblast differentiation probability	30%	Increased fibroblast differentiation or myofibroblast recruitment in the presence of activated inflammatory cells increased to 50% in disease.
	Proliferation	Fibroblast proliferation increased by inflammatory and epithelial cells	Fibroblast growth rate	132 iterations	Animal models indicate that lung fibroblasts divide every 5.5 days (132 iterations in model), which reduces to 2 days (48 iterations in model) in the animal model of asthma (25).
	Apoptosis	Myofibroblast survival supported by activated inflammatory cells	Myofibroblast apoptosis	5 cells	The threshold number of inflammatory cells below which myofibroblast apoptosis was induced, which was reduced to 2 in disease.

759

760 **Table 1. Agents, Rules and Model.** The computational airway model rule-set, parameters, and

761 which parameters were altered to observe airway remodeling.

762 **Figure legends**

763

764 **Fig. 1. Increased ASM mass in asthma is reduced by fevipiprant. A**. Representative
765 photomicrograph of a bronchial biopsy from a participant with severe asthma in the fevipiprant
766 (DP_2 antagonist) trial, showing increased ASM (brown stained α-SMA), disrupted epithelium
767 and lamina propria. **B**. ASM mass, as measured by percentage α-SMA positive area, in
768 bronchial biopsies from asthmatic subjects pre- and 12 weeks post-treatment with fevipiprant
769 (n=14) or placebo (n=13). A two-tailed paired t-test was used for within group comparisons
770 (p=0.022 and p=0.522), and a two-tailed unpaired t-test was used to compare the difference in
771 ASM mass observed following treatment with fevipiprant to that seen in the placebo group
772 (p=0.034).

773

774 **Fig. 2. Computational model-based investigation of interactions between airway**
775 **inflammation and ASM mass. A.** The mean time course from 6 simulations of the response
776 to epithelial injury (50% denudation at time zero) over 180 days showing increased eosinophil
777 numbers, ASM mass and persistent epithelial damage in the model of airway remodelling in
778 asthma versus resolution of the epithelial injury, eosinophil numbers and persistently low ASM
779 mass in the healthy control model ($p<0.01$ for comparisons of each parameter over time
780 between the patient model versus healthy control model, two-tailed unpaired t-tests). **B.**
781 Predicted reduction in eosinophil number over 180 days following reduction in eosinophil
782 recruitment or increase in eosinophil apoptosis in the computational model (n=5 simulations).
783 **C.** Relative change in the ASM mass percentage at 180 days, predicted as a consequence of
784 results in panel B (n=5 simulations).

785

Fig. 3. ASM cells express functional DP_2. A. Representative photomicrograph of DP_2 staining in bronchial biopsies from a subject with severe asthma (inset: isotype control). **B.** Quantitative PCR cycle threshold values for expression of ASM DP_2 mRNA vs the 18S ribosomal RNA housekeeping gene *RNA18S5*, mean DP2 Ct (threshold cycle) [95% CI]: 27.9 [26.1 - 29.8], n=7. **C.** Example histogram of DP_2 expression (black trace) in ASM cells by flow cytometry versus isotype control antibody (grey trace); fold increase in geometric mean fluorescence intensity (GMFI) of anti-DP_2 antibody/isotype control antibody [95% CI]: 1.3 [1.2 - 1.4], n=15 donors, $p<0.001$, two-tailed paired t-test against isotype control. **D.** Representative photomicrographs (x20 magnification) showing ASM α-SMA expression (green, left hand panel;isotype control antibody, inset) and ASM DP_2 expression (red, right hand panel; isotype control antibody, inset) by immunofluorescence staining; nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI, blue). **E.** F-actin polymerisation in primary human ASM cells (n=9 donors) in response to DK-PGD_2 treatment or Dulbecco's Modified Eagle's Medium (DMEM) containing 50% FBS as a positive control; geometric mean AUC DK-PGD_2 DR [95% CI]: 46 [25 – 104] x 10^2; p=0.01, one sample t-test against a hypothetical value of zero. **F.** Intracellular calcium (Ca^{2+}_i) elevation in primary human ASM cells (n=6-9 donors) in response to DK-PGD_2 treatment or ionomycin (1.5 μg/ml) as a positive control; geometric mean AUC DK-PGD_2 DR [95% CI]: 130 [78 – 230] x 103; p=0.002, one sample t-test against a hypothetical value of zero. Data are plotted as mean ± sem. Two-tailed paired t-tests were performed to compare each condition with its vehicle control; * $p < 0.05$, except FBS where Wilcoxon matched pairs sign ranked test was used, denoted by ^ $p < 0.05$.

Fig. 4. A DP_2 antagonist reduces ASM migration and recruitment of myofibroblasts and fibrocytes. All experiments were carried out in serum-free media. **A.** Shown are data for the wound closure after 24h of ASM cells that had been grown in monolayers and then wounded

by scratching with a pipette tip followed by incubation with different treatments for 24h: DP_2 agonist DK-PGD_2 (n=4-5 donors), the DP_2 antagonists fevipiprant (n=8 donors), CAY10471 (n=6-8 donors) and OC000459 (n=7 donors), or DMEM culture medium containing 10% FBS as a positive control. Two-tailed paired t-tests were performed to compare each condition with its vehicle control; * $p<0.05$ vs vehicle control. Data are expressed as mean ± sem. **B.** Representative photographs of ASM monolayers wounded by scratching with a pipette tip after 24h (vehicle control for fevipiprant, 500 nM fevipiprant, DMEM containing 10% FBS control (upper panel); vehicle control for CAY10471 and OC000459, 100 nM CAY10471 and 100 nM OC000459 (lower panel), black lines represent the wound edge at 0h, scale bar = 250 µm. **C.** PGD_2 release by unwounded and wounded ASM cells after 24h; p=0.02, Wilcoxon matched pairs signed rank test, n=10 donors. Data are expressed as mean ± sem. **D.** Representative flow cytometry traces of isotype control antibodies (grey traces) versus α-SMA expression (left hand panel, black trace, mean percentage fibrocyte population positive for α-SMA expression [95% CI]; 97 [93 – 100] %, n=4 donors) and DP_2 expression (right hand panel, black trace, GMFI fold difference DP_2 antibody/isotype control antibody [95% CI]; 1.6 [1.3 – 2]; p=0.0064, two-tailed paired t-test against isotype control antibody, n=6 donors) by fibrocytes. **E.** Correlation between change in myofibroblast number in the lamina propria and absolute change in ASM mass as a percentage of the total biopsy area (fevipiprant: black circle, n=14, placebo: black triangle, n=13), Spearman r [95% CI]: r=0.347 [-0.050-0.649]; p=0.076. **F.** Correlation between change in fibrocyte number in the lamina propria and absolute change in ASM mass as a percentage of the total biopsy area (fevipiprant: black circle, n=12, placebo: black triangle, n=13), Spearman r [95% CI]: r=0.538 [0.169-0.774]; p=0.006. **G.** Predicted relative reduction in percent ASM mass at 180 days in the computational model as a consequence of reduced myofibroblast recruitment (30-50%) in combination with a 40% reduction in eosinophil recruitment in the computational model (n=5 simulations).