ORIGINAL ARTICLE

Gene Expression Profile of Ovalbumin-Induced Lung Inflammation in a Murine Model of Asthma

R Malik,[1,*] P Priyadarsiny,[1,*] R Shirumalla,[2] R Soni,[3] A Ray,[2] K Saini[1]

[1]Department of Biotechnology, New Drug Discovery Research, Ranbaxy Research Laboratories, Gurgaon, India
[2]Department of Pharmacology, New Drug Discovery Research, Ranbaxy Research Laboratories, Gurgaon, India
[3]PREMAS Biotech, Gurgaon, India

Abstract

Background: Asthma is a chronic inflammatory disease that is associated with airway hyperresponsiveness, tissue remodeling, and airway obstruction, and that involves coordinate expression of multiple inflammatory genes in the lungs.
Objective: To evaluate the gene expression pattern in a mouse model of asthma and assess the effect of a new drug, R142571, on the gene expression profile.
Methods: Lung tissue from ovalbumin-sensitized mice was used to examine gene expression on the CodeLink oligonucleotide mouse 20 K bioarray platform. Data were validated for some genes by semiquantitative reverse-transcriptase polymerase chain reaction.
Results: Of the 19 736 genes represented on the microarray, expression of 378 genes was differentially regulated (215 upregulated and 163 downregulated), with at least a 2-fold change in expression ($P < .05$). The differentially regulated transcripts included genes known to be involved in several different biological processes, including signaling, DNA-dependent transcriptional regulation, immune response, proteolysis, and peptidolysis. Cluster analysis of the differentially regulated genes showed that at least 16 were downregulated by R142571 treatment at both of the doses used (1 and 10 mg/kg). In addition, 46 and 29 genes were downregulated at doses of 10 mg/kg and 1 mg/kg, respectively, as compared to the animals treated with vehicle.
Conclusion: The cytokine expression pattern in our data, suggests that the murine model exhibits a predominantly T helper 2-type response, as observed in asthmatic human subjects. Based on this study, we suggest that this mouse model would be an appropriate system for screening new drug molecules for treatment of atopic asthma.

Key words: Ovalbumin. Microarray. Lung. Cytokines. Asthma.

Resumen

Antecedentes: El asma, una enfermedad inflamatoria crónica, se relaciona con la hiperreactividad bronquial, la modificación de los tejidos y la obstrucción de las vías respiratorias, y esto implica una expresión coordinada de múltiples genes inflamatorios en los pulmones.
Objetivo: El objetivo fue evaluar el patrón de expresión génica en un modelo murino de asma y evaluar el efecto de un nuevo fármaco, el R142571, en el perfil de expresión génica.
Métodos: Se utilizó tejido pulmonar de ratones sensibilizados a la ovoalbúmina para examinar la expresión génica en una microarray de 20 K de oligonucleótidos murinos CodeLink. Se validaron los datos para algunos genes mediante la reacción en cadena semicuantitativa de la polimerasa retrotranscriptasa.
Resultados: De los 19. 736 genes representados en la microarray, se reguló diferencialmente la expresión de 378 genes (215 aumentados y 163 rebajados), con al menos el doble de cambios en la expresión ($P < 0,05$). Los transcritos regulados diferencialmente fueron genes de los que se conoce que están implicados en diversos procesos biológicos diferentes, como la señalización, la regulación transcripcional dependiente de DNA, la respuesta inmunitaria, la proteólisis y la peptidólisis. El análisis por grupos de los genes regulados diferencialmente mostró que al menos 16 se encontraban rebajados por el tratamiento de R142571 en las dos dosis que se utilizaron (1 y 10 mg / kg). Además,

*These authors contributed equally to the study

46 y 29 genes descendieron a dosis de 10 mg/kg y 1 mg/kg, respectivamente, como se comparó con animales tratados con vehículo. *Conclusión:* El patrón de expresión de la citocina según nuestros datos sugiere que el modelo murino muestra predominantemente 2 tipos de respuesta de linfocitos T cooperadores, tal y como se ha observado en personas asmáticas. De este modo, basándonos en este estudio, sugerimos que este modelo murino podría ser un sistema apropiado para la detección selectiva de moléculas de nuevos fármacos para el tratamiento del asma atópica.

Palabras clave: Ovoalbúmina. Microarray. Pulmón. Citocinas. Asma.

Introduction

Asthma is characterized by airway hyperresponsiveness, reversible airflow obstruction, and bronchoconstriction [1]. The majority of these symptoms are due to allergic inflammation, which is further characterized by the presence of mast cells, basophils, eosinophils, monocytes, and T helper (T_H) 2 lymphocytes. The allergic inflammatory response is regulated largely by T_H2 cytokines such as interleukin (IL) 4, IL-5, and IL-13. Analysis of lung tissue biopsies from patients with asthma has revealed that chronic airway inflammation is also associated with lung remodeling. These chronic changes include epithelial shedding and hypertrophy, hyperplasia and metaplasia of submucosal mucus glands and smooth muscle cells, and fibrosis [2-4]. Multiple mediators are produced in asthma, including lipid mediators, inflammatory peptides, chemokines, cytokines, and growth factors [5]. Most of the inflammatory proteins produced in asthmatic conditions are regulated by increased gene transcription controlled by proinflammatory transcription factors, such as nuclear factor κB (NF-κB) and activator protein-1 (AP-1), that are activated in asthmatic airways [6].

Currently, the most widely used experimental animal model for research into asthma is the mouse [7]. The key contribution of $CD4^+$ T-lymphocytes to the pathogenesis of asthmatic inflammation and the potentially crucial roles of T_H2 cytokines have been highlighted by several studies in this model system [8]. However, in order to use these models to test the efficacy of new therapies, a greater understanding of the molecular mechanisms underlying the experimental induction of asthma will be required. One approach to this problem is the use of DNA microarrays to assay the expression of thousands of genes simultaneously, allowing us to delineate the signal transduction pathways involved in asthma models.

The objectives of this study were to characterize changes in gene expression associated with the early response to ovalbumin challenge and to evaluate the effects of R142571, a novel anti-asthma drug, on the gene expression profile in this murine model of asthma.

Materials and Methods

Ovalbumin-Induced Mouse Model of Asthma

Female BALB/c mice aged 4 to 6 weeks were sensitized intraperitoneally with 10 μg of ovalbumin with aluminium hydroxide as adjuvant. Five mice were assigned to 4 different groups (control, ovalbumin-challenged without drug treatment, and ovalbumin-challenged animals treated with 1 mg and 10 mg of the drug). With the exception of control mice, all animals were treated with an ovalbumin aerosol once a day for 5 consecutive days. Those mice were then treated with an oral dose of vehicle or R142571 (1 or 10 mg/kg) 2 hours before ovalbumin challenge for 5 consecutive days. In parallel, control animals were treated with phosphate buffered saline (PBS).

To ensure proper allergen challenge, airway hyperresponsiveness of mice was assessed by noninvasive whole body plethysmograph, 4 hours after the last ovalbumin challenge. This involves placing the mouse in an airtight chamber and measuring breathing parameters. Five mice were sacrificed 4 hours after the final ovalbumin challenge. Whole pulverized mouse lung was flash frozen in liquid nitrogen and stored at –80°C until further analyzed.

RNA Extraction and Quantitation

The frozen lung tissue was homogenized in liquid nitrogen in an autoclaved pestle and mortar. The homogenized tissue was then allowed to thaw in extraction buffer prior to extraction of total RNA using the RNAeasy kit according to the manufacturer's instructions (Qiagen, Valencia, California, USA). RNA was quantified spectrophotometrically and purity was further assessed by gel electrophoresis.

Microarray Hybridization and Data Analysis

A microarray experiment was performed using the 4 experimental groups of mice. Three replica slides were hybridized with the same pool of RNA within each group. The CodeLink oligonucleotide mouse uniset 20K (from Amersham Biosciences, now part of GE health care, Piscataway, New Jersey, USA) was used in all experiments. Equal amounts of RNA were pooled from 5 mice in each treatment group and used to prepare the target according to manufacturer's protocol. cDNA synthesis, in vitro transcription, and the production of a biotin-labeled RNA probe for oligonucleotide arrays were performed as described by the manufacturer. The biotinylated cRNAs were hybridized to 12 CodeLink arrays. The hybridization mixture contained 10 μg of fragmented sample cRNA together with defined amounts of control bacterial spikes. The slides were scanned with a ScanArray Express microarray scanner (Perkin Elmer, Waltham, Massachusetts, USA) and the image was quantified with Codelink Expression

software (version. 4.0). Raw intensities were normalized using the global median for each chip and quantified data was further mined, analyzed, and visualized with Spotfire (version 8.0; Tibco, Palo Alto, California, USA). Statistical analysis was performed by analysis of variance using Spotfire.

TaqMan Validation Assay

Semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using Applied Biosystems TaqMan technology on ABI 7900HT. RNA was reverse transcribed with Multiscript reverse transcriptase (Applied Biosystems, Foster City, California, USA). Aliquots of each dilution were amplified with Amplitaq DNA polymerase. Cycling profiles included an initial 30-second denaturation step at 94°C followed by 2 step cycles of 94°C for 15 seconds and 60°C for 25 seconds, and completed with a final incubation at 72°C for 7 minutes. Derived Ct values were normalized against the constitutively expressed 18S RNA mRNA. Primers and probes were obtained from Applied Biosystems as assays on demand for several target genes including *IL4, IL5, IL13, IL2,* and *interferon (IFN)-γ.*

Results

Airway Hyperresponsiveness in Ovalbumin-Induced Murine Asthma

Measurement of early airway hyperresponsiveness by plethysmography showed that sensitized animals had significantly greater airway responsiveness to ovalbumin than control animals (Figure 1). Pretreatment of the mice with 2 different doses of R142571 (1 mg/kg and 10 mg/kg) led to a considerable reduction in airway hyperresponsiveness (Figure 1). These data indicate that measurement of airway hyperresponsiveness to inhaled ovalbumin by plethysmography is a useful indicator of airway hyperresponsiveness after allergen sensitization in mice.

Differential Gene Expression in a Mouse Model of Asthma

The gene expression profile of mouse lung tissue was determined 4 hours after the last challenge in animals treated with PBS, ovalbumin alone, and R142571 (1 mg/kg and 10 mg/kg dose) plus ovalbumin. Asthma-related transcripts were identified by comparing the expression profile for mice treated with PBS with those treated with ovalbumin alone. Three-hundred seventy-eight annotated genes on the CodeLink Uniset I mouse 20K oligonucleotide array showed at least a 2-fold difference in expression between PBS-treated and ovalbumin-treated mice ($P < .05$). Out of 378 annotated genes, 215 were upregulated and 163 were downregulated. These differentially expressed genes accounted for 1.9% of the 19 736 genes spotted on the microarray.

Ovalbumin challenge triggered the upregulation of inflammatory pathways including cytokine-cytokine receptor interaction and MAP signaling pathways. Differentially regulated transcripts belonged to genes involved in several biologically diverse processes, including chemotaxis, inflammatory response, immune response, transcription, DNA-dependent regulation of transcription, proteolysis, and peptidolysis. In addition, other important genes such as

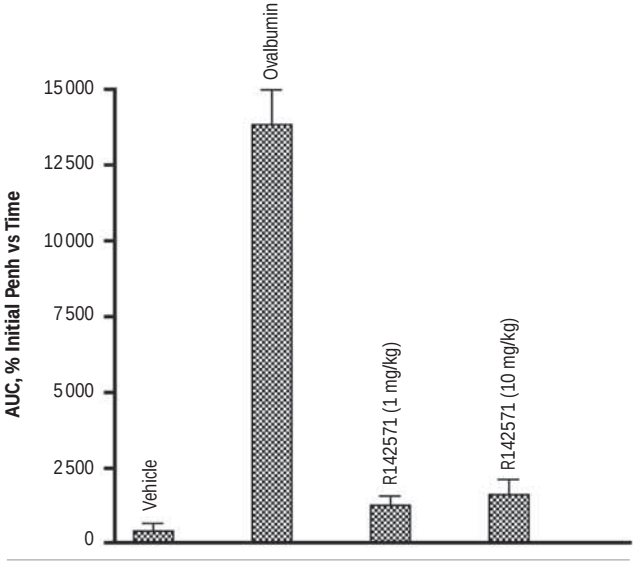

Figure 1. Effect of ovalbumin exposure on early airway response in Balb/c mice expressed as the area under the curve (AUC) for Penh (index of airway resistance) vs time. Curves were plotted for mice sensitized and challenged with ovalbumin and treated with R142571 at 2 doses. Bars show the mean of 5 animals. Whiskers show the SEM. Asterisks indicate $P < .05$ compared with the group treated with ovalbumin alone (1-way analysis of variance followed by Dunnett's multiple comparison test).

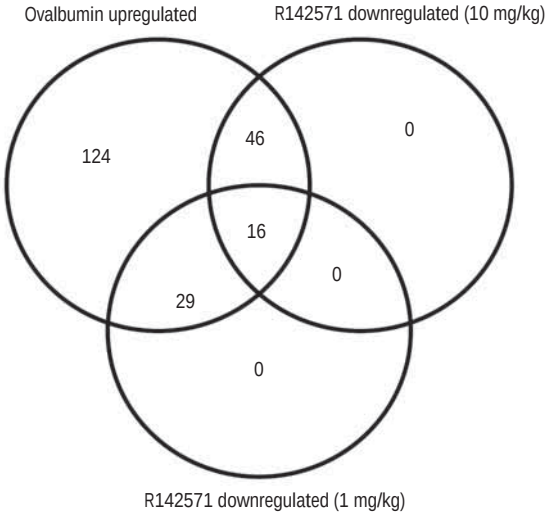

Figure 2. Venn diagram showing the number of genes upregulated in response to ovalbumin compared with those downregulated as a result of R142571 treatment. The intersection between the 3 circles of the Venn diagram contains genes that are commonly downregulated at both doses (1 mg/kg and 10 mg/kg) of R142571.

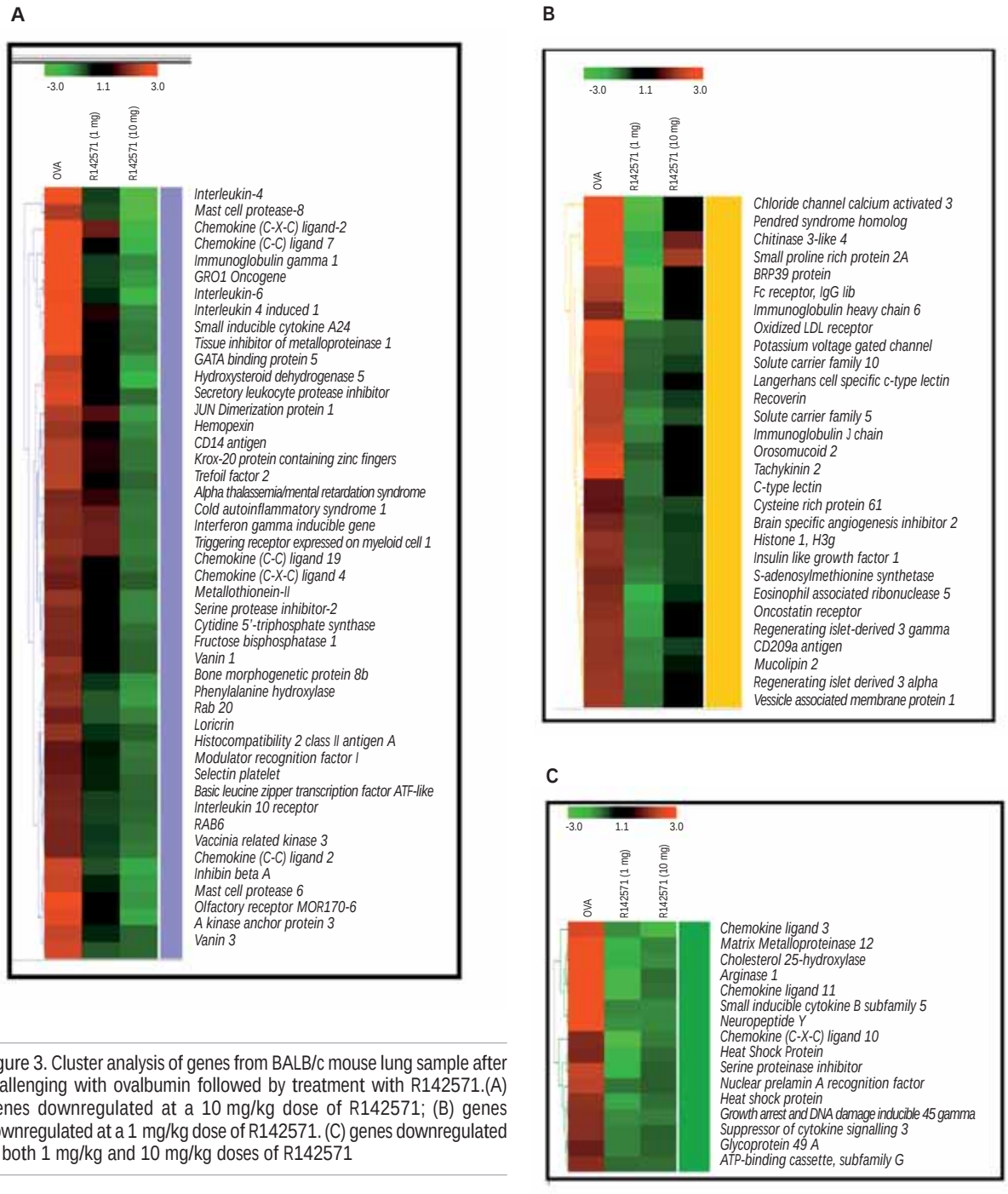

Figure 3. Cluster analysis of genes from BALB/c mouse lung sample after challenging with ovalbumin followed by treatment with R142571.(A) Genes downregulated at a 10 mg/kg dose of R142571; (B) genes downregulated at a 1 mg/kg dose of R142571. (C) genes downregulated at both 1 mg/kg and 10 mg/kg doses of R142571

chloride channel calcium activated (*Clca3;* 78 fold), *Arginase I* (*Arg* I; 10 fold), *matrix metalloproteinase 12 (MMP-12*; 9 fold), *tissue inhibitor of metalloproteinase* 1 (*TIMP-1*; 9 fold), *selectin platelet* (*Selp*; 2 fold), *suppressor of cytokine signaling* 1 (*Socs*1; 3.5 fold), and *suppressor of cytokine signaling 3* (*Socs 3*; 2.6 fold) were found to be upregulated. Selection of differentially expressed genes based on a 2-fold cutoff neglected overexpression of *nuclear factor of kappa light polypeptide gene enhancer in B-cells* 2 (*NF-κB*, 1.5 fold), *histone acetyltransferase* (1.7 fold), and *CCAAT/enhancer binding protein delta* (*C/EBPδ*; 1.7 fold), which may be equally important in the etiology of asthma.

R142571 treatment suppressed certain genes upregulated in response to ovalbumin. Out of 215 upregulated genes, 91 were downregulated at least 2 fold in response to treatment with either 1 mg/kg or 10 mg/kg R142571 (Figures 2 and 3). Statistical cluster analysis of upregulated genes revealed 4 major clusters: (1) genes that were not downregulated by

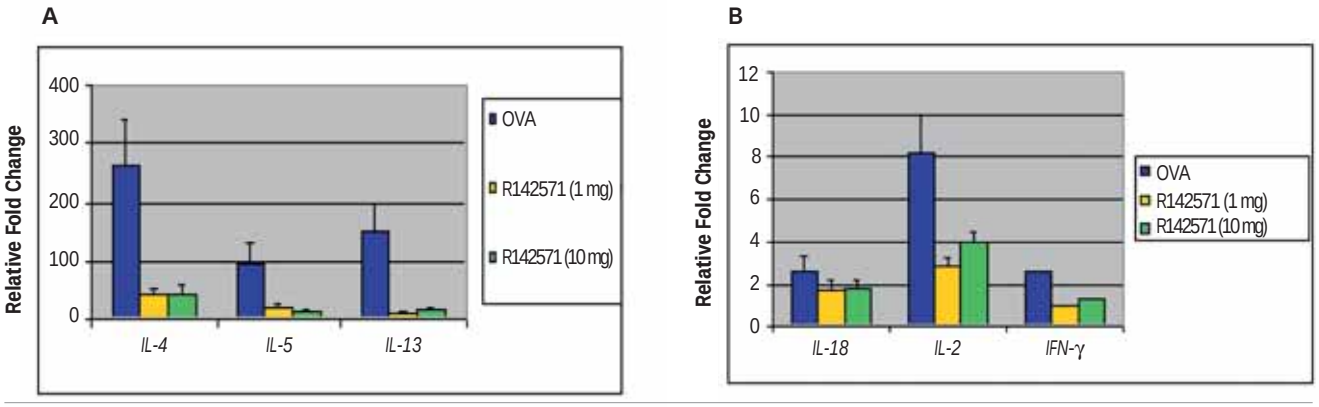

Figure 4. Transcriptional response of T helper (T_H) 1 and T_H2-specific genes in BALB/c mouse lung after ovalbumin (Ova) challenge followed by drug treatment as detected with semi-quantitative real time polymerase chain reaction. (A) Expression level of *interleukin (IL)-4*, *IL-5* and *IL-13*. (B) Expression level of *IL-18*, *IL-2*, and *interferon-γ*

either dose of R142571; (2) genes that were exclusively downregulated by the 1 mg/kg dose of R142571; (3) genes that were exclusively downregulated by the 10 mg/kg dose of R142571; and (4) genes that were downregulated by both doses of R142571 (Figure 2). R142571 at 10 mg/kg suppressed the majority of genes that were upregulated at least 2 fold in asthmatic mice (Figure 3A). The major genes involved in inflammatory pathways were *IL-4, IL-6, IL-10R, mast cell protease 6* (*MCP-6*), *MCP-8, CXCL-2* (*MIP-2ß*), *CCL7, CCl19* (*MIP-3ß*), CXCL4 (*platelet factor-4*), *CCL2* (*MCP-1*), *TIMP-1, GATA binding protein-5, CD-14 antigen, selectin platelet*, and *basic leucine zipper transcription factor ATF like*. In contrast, R142571 at 1 mg/kg only suppressed upregulation of 29 genes (Figure 3B). Sixteen genes were downregulated at both doses of R142571, including *CCL-3* (*MIP-1 α*), *CCL11 (Eotaxin), CXCL10* (*γIP10*), *ArgI, MMP12, SOCS-3* (Figure 3C).

Semiquantitative PCR Analysis

Overall, these results indicate that the transcriptional responses following ovalbumin challenge in this mouse model may underlie phenotypic changes believed to be important in the development of human allergic asthma. Real-time PCR data clearly showed T_H2-type inflammation when compared to T_H1-type cytokines (Figure 4). In comparison to the T_H1 cytokines *IL-2* and *IFN-γ* (8 and 2.6 fold change in expression, respectively) the expression of T_H2 cytokines *IL-4*, *IL-5*, and *IL-13* was upregulated 262, 95, and 150 fold, respectively, thus confirming that this murine model is skewed towards a T_H2-mediated response. After treatment with R142571, the expression level of the cytokines was significantly reduced (Figure 4).

Discussion

In this study, we demonstrated the presence of early airway hyperresponsiveness or bronchoconstrictive responses induced by ovalbumin. Furthermore, our data clearly show that the increase in airway hyperresponsiveness caused by ovalbumin is suppressed upon treatment with our new chemical entity, R14271. Ovalbumin has been shown previously to induce asthma in mice [10]. Many genes observed in this study have been previously shown to be associated with asthma, eosinophilia, airway hyperreactivity, or epithelial cell metaplasia [11,12]. The observed upregulation of *NF-κB, histone acetyltransferase*, and *C/EBP* confirms previous findings [6,13] and supports a role for these genes in orchestrating the inflammatory response in asthma. For instance, acetylation of histone residues by histone acetyltransferase allows transcription factors to bind more readily and could therefore be involved in upregulating transcription of inflammatory genes [6-14].

Our data showed R142571 to be a potent inhibitor of lung inflammation. At the 2 doses tested, the drug suppressed the expression of several inflammatory genes involved in asthma. It also inhibited the expression of *NF-κB, basic leucine zipper transcription factor ATF like*, and *C/EBP*, at both the doses tested. The possible mechanism by which R142571 suppressed expression of genes involved in asthma could be through the inhibition of the transcription factor NF-κB, which is a major regulator of inflammatory genes including cytokines, chemokines, inflammatory enzymes, adhesion molecules, and inflammatory receptors [15]. R142571 is a selective small molecule inhibitor, and even at low doses it could be used as an effective treatment compared to inhaled corticosteroids, the only therapy that suppresses inflammation in asthmatic airways by inhibiting the effect of proinflammatory transcription factors such as AP-1 and NF-κB. However, inhaled corticosteroids are marginally effective in chronic obstructive pulmonary disease and may be of lesser benefit in viral exacerbations of asthma [16,17]. In most cases of asthma, disease severity or steroid insensitivity mean that patients respond poorly to inhaled treatments, leading to the possibility that higher doses are used with possible undesirable side effects [18,19]. In such cases, it has been reported that small molecule drugs are more selective and would be a better therapeutic option [18].

Our study showed that R142571 suppresses expression of *ArgI* significantly at both the doses tested. In an earlier study, *ArgI* activity was shown to be increased in mouse lung and in bronchoalveolar lavage fluid from human asthmatic patients [20]. Thus, by regulating the expression of *ArgI*, induction of airway hyperresponsiveness may be modulated. In addition to the genes involved in lung inflammation, genes involved in tissue remodeling such as *MMP-12* and *TIMP-1* were also overexpressed in ovalbumin-challenged mice. MMP-12 has a prominent role in tissue remodeling and destruction [21], and the present study shows that R142571 also inhibits the altered expression of *MMP-12* in this mouse model of asthma.

The gene expression profile of various cytokines in the present study showed greater upregulation of T_H2-type cytokines including *IL4, IL5*, and *IL13*, thereby indicating that the ovalbumin-challenged asthmatic mouse model may preferentially use the T_H2 pathway during the initial phases of the disease. The upregulation of *IL-4, IL-5*, and *IL-13* was found to be inhibited at both doses of R142571. Semiquantitative PCR studies further analyzed the upregulation of *IL-12* and *IL-18*, which have been shown previously to suppress type 2 responses and to exhibit proallergic effects [22].

The expression pattern of the cytokines suggests that the majority of murine models of allergic asthma exhibit a predominant T_H2-type response, as may also occur in human asthmatic individuals [23]. In other words, the $CD4^+$ T_H2 lymphocytes that recognize the antigen are activated and differentiate into a cell subtype that is characterized by the release of a certain range of cytokines, notably IL4, IL5, and IL13. In mice, the relevance of IL5 and IL13 in asthma has been described, and it has been pointed out that both mediators, as well as other T_H2 cytokines such as IL4, exert various modulatory effects on bronchial hyperreactivity, inflammatory infiltration of eosinophils, and remodeling of lung tissue [23-29]. An earlier study also showed that the closely linked *IL-4, IL-5*, and *IL-13* genes were coordinately regulated during human T_H2 differentiation [30]. *IL-4, IL-5*, and *IL-13* are closely linked on human chromosome 5 and murine chromosome 11 [31], and this has led to considerable speculation that they may be coordinately regulated. Initial work on murine T-cell clones demonstrated that T_H1 cells express *IL-2* and *IFN-*γ, whereas T_H2 cells express *IL-4, IL-5, IL-10*, and *IL-13* [29]. Enhanced levels of *IL-4, IL-5*, and *IL-13* mRNA have also been observed in asthmatic human subjects compared to healthy controls [30]. By directly blocking the signal transduction pathway of these cytokines, R142571 could be a more specific and safer therapy for the patient. The effect of the drug in blocking upregulation of *IL-4* and *IL-13* in an ovalbumin-induced murine model of asthma further highlights the potency of the drug.

In conclusion, we have demonstrated that the ovalbumin-induced mouse model of asthma shows significant alterations in lung gene expression profile compared with control animals. We have also shown that R142571 is a potent selective inhibitor that significantly reduces cytokine production and suppresses inflammation-related genes responsible for the allergic asthmatic phenotype.

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Dr Priyanka Priyadarsiny

Department of Cell Biology
Zydus Research Centre
Cadila Healthcare Ltd
Sarkhej-Bavla National Highway Nº 8 A
Near Nova Petrochemical Ltd
Moraiya, Ahmedabad 382210, Gujarat, India
E-mail: ppriyadarsiny@gmail.com