



Mepacrine treatment attenuates allergic airway remodeling segregated from airway inflammation in mice[☆]

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ABSTRACT

Asthma is a chronic airway disease characterized by increased airway hyperresponsiveness, airway inflammation, and airway remodeling including collagen deposition in subepithelial regions. We have shown earlier that mepacrine has anti-inflammatory activity and decreased the features of airway remodeling in a subacute model of asthma, when administered during the inflammatory phase. But it was not clear whether the reduction of airway remodeling by mepacrine was a direct effect or indirectly related to the reduction in the airway inflammation. In this study, we determined the effect of mepacrine on airway remodeling and airway hyperresponsiveness (AHR) in a chronic model of asthma which showed the features of airway inflammation in the initial stage (inflammation predominant stage) and airway remodeling with mild airway inflammation in a later stage (remodeling predominant stage). Mepacrine was administered only in the later stage that more accurately simulates human asthma, where airway remodeling already exists at the time of diagnosis. The remodeling predominant stage was associated with high levels of Th2 cytokines like IL-4 and IL-13, increase in the levels of profibrotic mediators such as arginase and TGF- β , and increased collagen deposition. These were efficiently attenuated by mepacrine treatment and led to a significant reduction in AHR. Thus, we conclude from this study that mepacrine has direct effects on established airway remodeling independent of its anti-inflammatory effects.

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1. Introduction

Asthma is a chronic airway disease characterized by variable airway obstruction, hyperresponsiveness, inflammation including airway eosinophilia, and mucus hypersecretion [1]. Inflammation is considered to be the central feature of asthma, and anti-inflammatory drugs along with bronchodilators form the mainstay of asthma therapy [2]. However, the inflammation theory, especially eosinophilic inflammation, has been found lacking in explaining the progressive morbidity associated with asthma [2–4]. The correlation between basement membrane thickening, which is predominantly due to collagen deposition, and eosinophilic airway inflammation was poor in asthmatic patients [5]. Also, the ability of anti-inflammatory therapy to prevent airway remodeling is modest [6–8]. These indicate not only the complex nature of the disease and also the necessity of the understanding airway remodeling.

We have shown that mepacrine attenuates asthmatic features in murine models of asthma [9,10]. In such models, inflammation precedes remodeling in a well-defined sequence. Mepacrine has been shown to have antioxidant properties and is a well-known anti-malarial drug [11,12]. We repurposed it for asthma due to its various pharmacological properties which are relevant to asthma. This approach was similar to efforts to repurpose other old drugs for asthma due to their new recognized activities [13,14]. The anti-asthmatic effects of mepacrine are presumably mediated through inhibition of PLA2 and TGF- β signaling [8,9]. Since these pathways participate both in inflammation and remodeling, and we had administered mepacrine during the development of allergic inflammation, the utility of mepacrine as a drug to target airway remodeling was unclear. However, it is practically difficult to separate these events since the line to distinguish is not clear and also there is an involvement of common mediators in both events. We have recently generated a chronic model of asthma that shows features of airway inflammation in initial stage (inflammation predominant stage) and airway remodeling with mild airway inflammation in a later stage (remodeling predominant stage) [15]. The later stage is closer to the actual therapeutic scenario in humans where patients already have airway remodeling at the time of diagnosis. In this study, we use that segregation to determine the effects of mepacrine on structural changes by administering the drug right after the reduction in inflammation and also determine the effect of mepacrine on profibrotic mediators such as arginase and TGF-beta in this model.

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2. Materials and methods

2.1. Animals

Seven- to 8-week-old male BALB/c mice were obtained from Central Drug Research Institute, Lucknow, India and acclimatized for a week prior to starting the experiments. All animals were maintained as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, and the protocols were approved by the Institutional Animal Ethics Committee.

2.2. Grouping of mice

Mice were divided into three groups ($n=6$) and were named according to sensitization/challenge/treatment: SHAM/PBS/VEH (normal controls, VEH-vehicle, water), OVA/OVA/VEH (allergic controls, OVA, chicken egg ovalbumin, Grade V, Sigma St. Louis, MO), OVA/OVA/MEP (MEP, mepacrine, Sigma St. Louis, MO). A 1-mg/kg dose MEP was given orally from days 36 to 50 twice a day (12 hrs interval between two doses) in the volume of 10 μ l [8,9].

2.3. Sensitization, challenge and treatment of mice

Mice were sensitized and challenged as shown in Fig. 1, briefly, mice were injected with 50 μ g OVA in 4 mg aluminum hydroxide or only 4 mg aluminum hydroxide by intraperitoneal route on days 0, 7, and 14, and challenged with 1.5% OVA in PBS or PBS alone for 4 weeks on alternate days.

2.4. Lung histopathology

On day 51, each mouse was sacrificed; lungs were harvested [16]. Formalin fixed, paraffin embedded, lung tissue sections were stained with Periodic acid-Schiff and Masson's Trichrome stainings and quantitative morphometry was performed as described earlier [16,17].

2.5. Measurements of IL-4, IL-5, IL-13, IL-10 and TGF- β 1 levels in the lung

ELISA of IL-4, IL-5, IL-10 and TGF- β 1 (BD biosciences) and IL-13 (R&D systems) were performed as per the manufacturer's instructions.

2.6. Arginase activity assay in lung cytosols

Lung cytosols were prepared and protein estimation was performed by bicinchoninic acid assay (Sigma Chemical Co) [16]. Arginase activity in lung cytosols was determined as described earlier [17] with little modifications. Briefly arginase assay medium consists of 30 mM arginine and 100 mM MnCl_2 was added to 50 μ g lung

cytosol and this mixture was incubated at 37 °C for 20 min followed by terminating the reaction by addition of HClO_4 . Urea was then measured with neutralized extracts at 340 nm with urea assay kit (Span Diagnostics Ltd, India).

2.7. Western blot for Arginase I

Ten percent SDS-PAGE was performed to separate proteins of lung cytosols, transferred onto PVDF membranes (Millipore Corp, USA), and transferred membranes were blocked with blocking buffer (3% skim milk in PBST (phosphate buffered saline with tween 20)), incubated with Arginase I antibody (1: 500, Santa Cruz, CA), followed by HRP conjugated anti-goat secondary antibody (1:1000, Sigma USA) and detected with DAB- H_2O_2 (Sigma, USA). α -tubulin was used as a loading control.

2.8. Total lung collagen

The total collagen content of the lung homogenates (prepared by homogenizing lung tissue in PBS, centrifuged at 10,000 rpm for 30 minutes, total protein was estimated in supernatant) was estimated with sircol collagen assay kit (Biocolor life science assays, UK) as per the manufactures instructions.

2.9. Airway hyperresponsiveness (AHR) measurement

AHR to methacholine (MCh, Sigma) was determined by both double chamber whole body plethysmography, (PLY 3351, Buxco electronics) and flexivent (Scireq, Canada) respectively as described earlier with some modifications [17,18].

2.10. Statistical analysis

Data are expressed as means $\pm$ standard error of the mean (SEM). Statistical significance was set at $p \leq 0.05$. ANOVA with post-hoc correction was used to compare group-wise data.

3. Results

3.1. Mepacrine treatment reduces Th2 cytokines in mouse model of asthma in which airway remodeling was segregated from airway inflammation

To determine the effect of mepacrine on airway remodeling in a chronic model of asthma, which showed the modest reduction of airway inflammation, we used a mouse model of asthma with features of airway inflammation in initial stage (inflammation predominant stage) and airway remodeling with mild airway inflammation in a later stage (remodeling predominant stage) [15]. Since it is known that Th2 cytokines are necessary for the development of airway

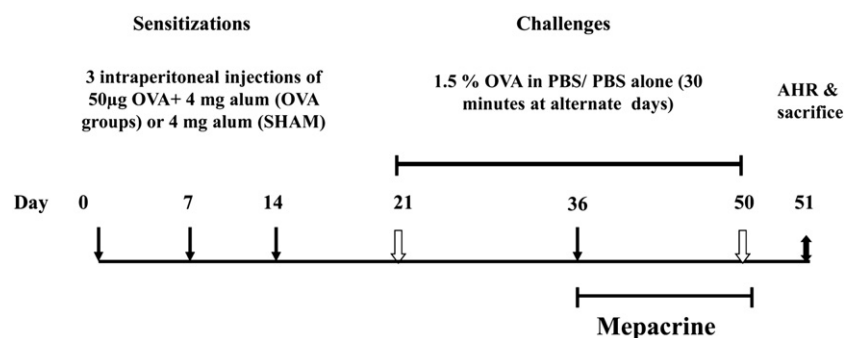


Fig. 1. Schematic diagram of experimental protocol. Mice were immunized by intraperitoneal injections of ovalbumin (OVA) on days 0, 7, and 14 and exposed to 1.5% OVA aerosol in alternative days (30 min daily) from days 21–50. To evaluate the effect of mepacrine (MEP) on asthma features such as airway remodeling and AHR, mice were administered MEP orally from days 36 to 50. On day 51, AHR was estimated and mice were sacrificed.

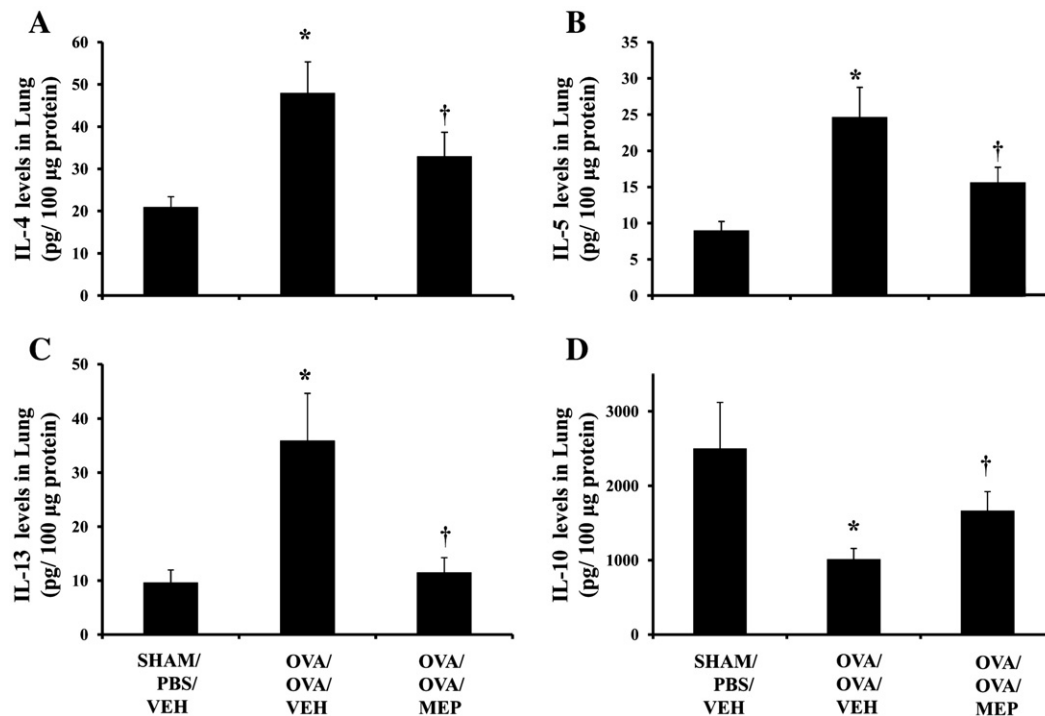


Fig. 2. MEP reduced the levels of Th2 cytokines in lung. OVA-sensitized and challenged mice were treated with MEP, lung homogenates were prepared, and IL-4 (A), IL-5 (B), IL-13 (C) and IL-10 (D) levels were determined by ELISA. * $P \leq 0.05$ vs. SHAM/PBS/VEH, † $P \leq 0.05$ vs. OVA/OVA/VEH group.

remodeling, we determined the levels of IL-4, IL-5 and IL-13 in lung tissue homogenates. As shown in the Fig. 2, the levels of these Th2 cytokines were increased in OVA/OVA/VEH as compared to SHAM/PBS/VEH. However, treatment of OVA challenged mice with MEP significantly reduced these cytokines (Fig. 2A, B and C). Interestingly, MEP treatment increased the levels of immunomodulatory cytokine IL-10 (Fig. 2D).

3.2. Mepacrine treatment reduces arginase and TGF- β_1

Since it is known that Th2 cytokines such as IL-4 and IL-13 induce arginase directly or indirectly through TGF- β_1 , we wanted to determine the effect of MEP on these profibrotic mediators. As shown in Fig. 3, OVA mice had increased activity and expression of arginase in lung cytosols. This was significantly reduced with MEP treatment. Furthermore, OVA mice showed increase in the levels of TGF- β_1 and MEP treatment reduced its levels in lung homogenates (Fig. 4A).

Since MEP treatment had reduced Th2 cytokines and pro-fibrotic mediators in this segregated model, we wanted to determine the effect of MEP on total lung collagen in this model. As shown in Fig. 4B, OVA/OVA/VEH mice showed an increase in total lung collagen compared to SHAM/PBS/VEH mice. However, MEP treatment reduced this significantly (Fig. 4B).

3.3. Mepacrine treatment reduces airway remodeling

To determine the effect of mepacrine on histological lung remodeling features such as mucous metaplasia and sub-epithelial fibrosis, lung sections were stained with Masson's trichrome and Periodic acid-Schiff stainings to estimate collagen deposition and mucous metaplasia respectively. As shown in Fig. 5A and B, there was dense deposition of collagen fibers around the bronchi and vessel and goblet cell metaplasia in OVA/OVA/VEH (II) mice compared to SHAM/PBS/VEH (I). MEP treatment reduced both collagen deposition and goblet cell metaplasia significantly (Fig. 5A (III) and B(III)).

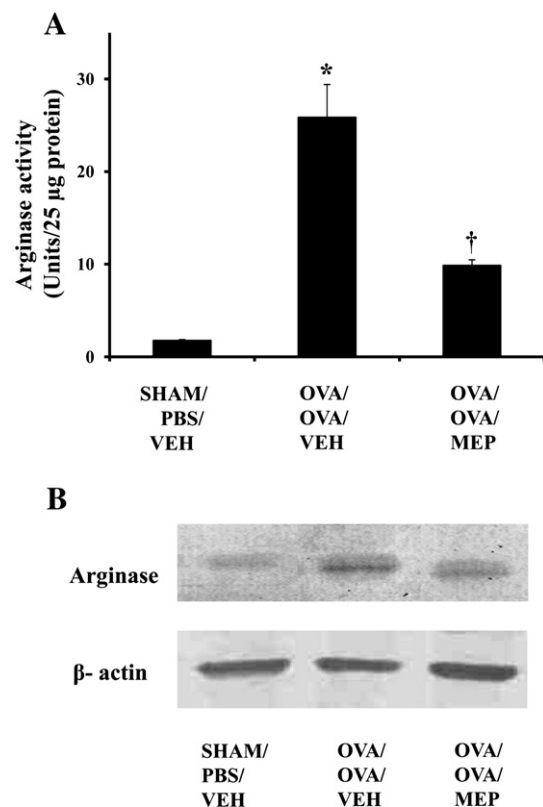


Fig. 3. MEP reduced the activity and expression of arginase in lung cytosol. Arginase activities (A) and Western blots for arginase I and (B) β actin were performed in lung cytosols as described in Materials and methods. * $P \leq 0.05$ vs. SHAM/PBS/VEH, † $P \leq 0.05$ vs. OVA/OVA/VEH group.

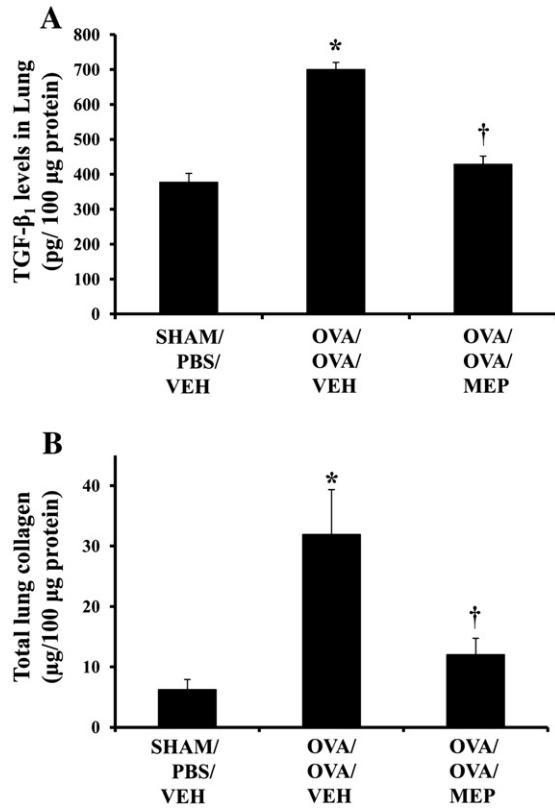


Fig. 4. MEP reduced the levels of TGF-beta and total collagen contents in lung. OVA-sensitized and challenged mice were treated with MEP and TGF-beta (A) and total collagen content (B) were estimated in lung homogenates as described in [Materials and methods](#). * $P \leq 0.05$ vs. sham/PBS/VEH, † $P \leq 0.05$ vs. OVA/OVA/VEH group.

3.4. Mepacrine treatment reduces airway hyperresponsiveness

To elucidate the effects of MEP on airway function in this chronic model, specific airway conductance and airway resistance were determined as described in [Materials and methods](#). As shown in [Fig. 6](#), there was significant reduction in percentage baseline sGAW and increase in airway resistance after methacholine (MCh) challenge in OVA/OVA/VEH mice compared to SHAM/PBS/VEH mice. However, MEP treatment improved the lung function by increasing sGAW ([Fig. 6A](#)) and decreasing airway resistance ([Fig. 6B](#)).

4. Discussion

Mepacrine, a known antimalarial drug, has been used in various inflammatory diseases, infections and autoimmune diseases for about eight decades [19]. The widespread activities of mepacrine are due to various reasons such as its immunomodulatory effects [19,20], and the involvement of PLA2 in various diseases [21]. We have previously shown for the first time that it also has in vivo anti-asthma property by reducing AHR, airway inflammation and subepithelial fibrosis via PLA2 and arginase pathways [9,10] using both acute and sub-acute models of asthma. In those studies, two issues remained unclear. First, it was not clear that the observed reduction in subepithelial fibrosis by MEP was the primary effect or secondary effect due to reduction in airway inflammation since we administered MEP during the development of airway inflammation. Second, it was not clear whether MEP would be effective in asthma when initiated after remodeling had started, which is more pertinent to human use. In this study, we used a chronic OVA model where we found gradual reduction of airway inflammation and gradual increase in structural changes upon repeated OVA exposures. Similar findings were also observed by other groups [22]. We focused on the effect of MEP in this model with the administration of the drug after initiation of airway remodeling, and found that it efficiently attenuated airway remodeling. Indeed, such studies are required for other available antiasthma drugs to find the direct effects in airway remodeling since there are reports suggesting

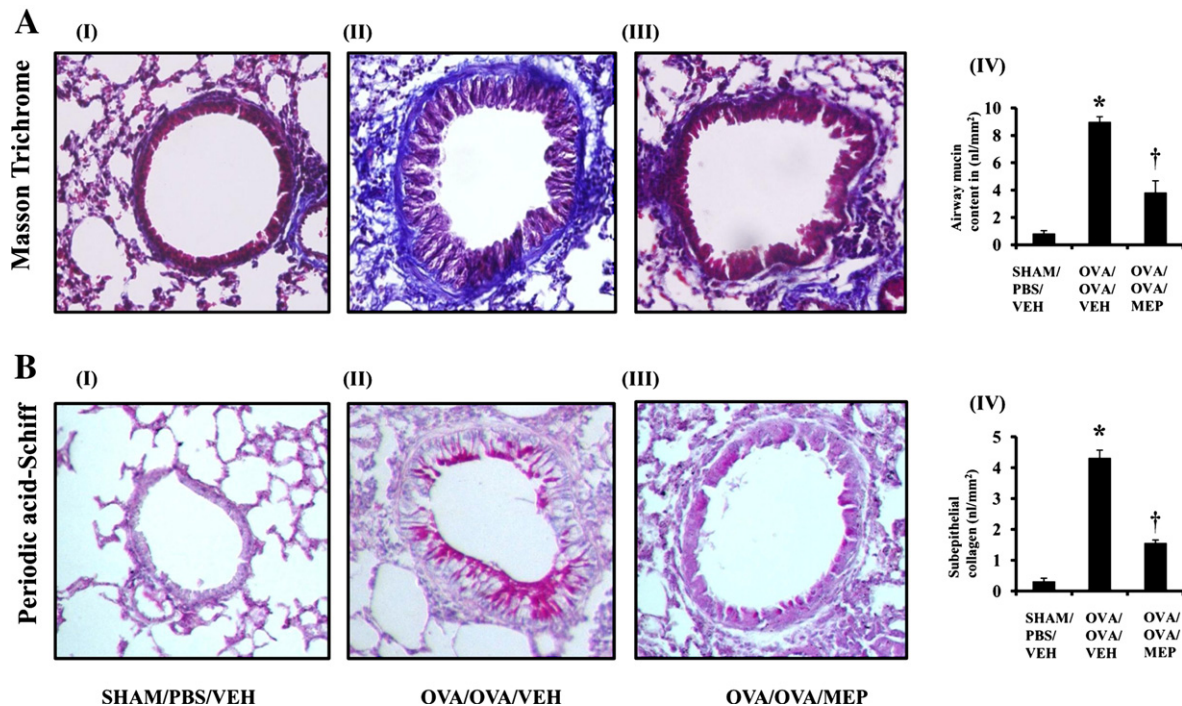


Fig. 5. MEP reduced airway remodeling changes. OVA-sensitized and challenged mice were treated with MEP and lung sections were stained with periodic acid-Schiff (A) and Masson's trichrome (B) stainings (I, II and III) to determine the effects of MEP on mucus metaplasia and subepithelial fibrosis, respectively and further analyzed by quantitative morphometry (IV). All the images were taken at 10 $\times$ magnification. * $P \leq 0.05$ vs. sham/PBS/VEH, † $P \leq 0.05$ vs. OVA/OVA/VEH group.

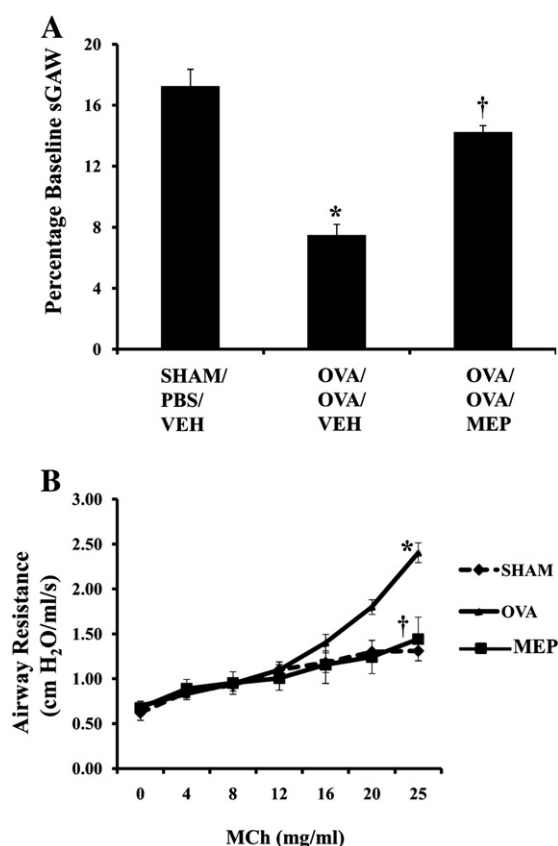


Fig. 6. MEP reduced airway hyperresponsiveness to methocholine. (A) Specific airway conductance (sGAW) was determined on day 51 using double chamber plethysmography and results were expressed as percentage change in sGAW with increasing concentrations of methocholine. (B) Airway resistance was determined using flexiVent and results were expressed in cm H₂O/ml/s with increasing concentrations of methocholine. * $P \leq 0.05$ vs. sham/PBS/VEH, † $P \leq 0.05$ vs. OVA/OVA/VEH group.

that airway remodeling may occur even before the development of symptoms [23] in contrast to earlier belief that airway remodeling is the end part of the disease.

Interestingly, we found that Th2 cytokines increased during the untreated remodeling phase although there was scarce inflammatory cells, indicating the possibility of other cellular sources of these cytokines; probably structural cells. Also it seems that these cytokines are sufficient to develop the structural changes since administration of recombinant IL-4 or 13 to wild type mice was sufficient to cause goblet cell metaplasia [24,25]. These evidences indicate that the symptoms of asthma may develop independent of cellular inflammation. In this study, MEP was found to reduce both IL-13 and IL-4. These Th2 cytokines stimulate bronchial epithelia to secrete TGF- β bypassing the need for inflammatory cells [26]. Again we found that MEP reduced the levels of TGF- β in lung.

Importantly, we found that mepacrine also reduced another profibrotic mediator, arginase along with significant reduction in lung collagen content. Earlier we had found that arginase is predominantly expressed in inflammatory cells present in perivascular and peribronchial regions. However, the source of arginase is not known in this model, and this needs further investigations. Arginase produces proline and polyamines from L-arginine, which leads to subepithelial fibrosis and smooth muscle hypertrophy respectively [27]. In our study, mepacrine administration did not affect health of mice, in line with many previous studies and its known use in humans. In conclusion, mepacrine reduces the features of airway remodeling with reduction in profibrotic mediators in a remodeling predominant model of asthma, indicating its possible anti-remodeling effect independent of airway inflammation and suitability for use as a drug post development of asthma.

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