Chapter 4

To study the effect of protease inhibitor in combination with kinase inhibitor in airway inflammatory disease
4.1. INTRODUCTION

Prevalence of allergic diseases is increasing and is a major public-health concern (Devereux, 2006). Allergen exposure triggers the allergic exacerbation in atopic individuals. A major group of allergens possess protease activity e.g. in house dust mite, insects, molds pollens etc. (Gupta et al., 2004; Bisht et al., 2004; Chua et al., 1988; Sudha et al., 2008). Proteases exaggerate the allergic reaction by disrupting epithelial barrier, activating immune cells to increase the level of inflammatory cytokines (Kale & Arora, 2014), inducing Th2 response (Goel et al., 2012; Kheradmand et al., 2002) and enhancing IgE synthesis (Post et al., 2014). Protease also induces bronchoconstriction (Proud et al., 1988; Tam & Caughey, 1990) and tissue remodeling (Zhu et al., 2013; Saw & Arora, 2014; Sommerhoff, 2001). Cockroach is common a source of air borne protease allergens (Sudha et al., 2008) and protease activity enhances allergic effect (Sudha et al., 2009; Arizmendi et al., 2011).

Protease induces various inflammatory mediators by activating protease activated receptors (Akers et al., 2000) and subsequently activating the signaling cascade, which involves multiple signaling molecules including kinases (Yu et al., 2010). Protease phosphorylates signaling kinases PI3K followed by ERK1/2 and p38 kinase (Rohani et al. 2010; Zeng et al., 2013; Zhang et al., 2014). Expression of protease induces inflammatory mediators was regulated by particular kinase or set of these kinases (Zeng, et al., 2013). P38 kinase is involved in inflammatory mediator secretion such as TNFα, IL-1β (Schieven, 2005). PI3K kinase and ERK1/2 kinase are involved in T cell activation, clonal expansion and subsequent inflammatory cytokine secretion (Egerton et al., 1996; Han et al., 2012). These inflammatory mediators are released through complex interplay of PI3K/ ERK1/2 / p38 kinase and hence inhibitors may have therapeutic potential in allergic diseases (Lee et al., 2006; Duan et al., 2005; Duan et al., 2004; Choudhury et al., 2002).

Kinase inhibitors have immense potential to lower allergen induced immune response and are emerging therapy in allergic diseases. Protease inhibitors are also considered as newer therapeutic option for the airway inflammatory diseases. Earlier studies have shown that a serine protease inhibitor AEBSF has therapeutic potential in airway inflammatory disease (Saw et al., 2012; Saw & Arora, 2015). It will be
worthwhile to target different pathway to enhance therapeutic potential (Bansal et al., 2014). Therefore, the present study was aimed to use serine protease inhibitor with signaling pathway inhibitors.

### 4.2. MATERIAL AND METHODS

#### 4.2.1. Animal experiment

Female BALB/c mice of 4-6 weeks were procured from NIN Hyderabad (India) and kept in animal house facility to get acclimatize. Animals were kept under 12 hour controlled light: dark cycle and fed chow based diet (*ad libitum*). The experimental protocol followed was approved by animal ethics committee of “CSIR-Institute of Genomics and Integrative Biology”.

Mice were divided into 6 mice per group and sensitized by 100 µg of cockroach extract by i.p injection on day 0, 7 and 14 and challenged with 5 µg of allergen intranasally on day 27, 28 and 29. One group of CE sensitized mice was treated intranasally with vehicle 5% DMSO (PBS) one hour before allergen challenge. Another group was treated with 30 µg of AEBSF (Sigma, St Louis, USA) in vehicle. Mice were also i.n administered; PI3K inhibitor LY294002 (iPI3K) (3mg/kg) (Sigma, St Louis, USA), ERK1/2 inhibitor U0126 (iERK1/2) (3mg/kg) (Sigma, St Louis, USA) and p38 kinase inhibitor SB203580 (ip38) (3mg/kg) (Sigma, St Louis, USA) in groups. Similarly three groups were administered with these signaling inhibitors along with AEBSF one hour before challenge. All intranasal treatments were given after anesthetizing the mice with isoflurane. The control group of mice were sensitized and challenged with PBS.

The dose for the iPI3K in present study was selected on the basis of previous study (Newcomb et al., 2008). An intraperitonial dose of 7.5-30 mg/kg of U0126 was used in ovalbumin induced mice model of airway disease (Duan et al., 2004) and even lowest dose was found effective in reducing inflammation. Droebner et al. (2011) showed 9.4 µg dose of iERK1/2 in lung reduced 50% progeny influenza virus in mice. In the present study, intranasal dose of 3mg/kg of iERK1/2 was given to the mice. A subcutaneous dose of 3mg/kg of ip38 was found effective in contact allergy in mice model (Schottelius et al., 2010) and was selected for present study.
4.2.2. Assessment of airway resistance

Airway resistance of mice was measured using FlexiVent TM ventilator (Scireq, Montreal, Que., Canada). Mice were anesthetized by intraperitoneal injection of Xylazine (10mg/kg) and pentobarbital (100mg/kg). Pressure transducer of ventilator was calibrated with 18 gauge cannula, at a pressure of 0 and 30 cm H$_2$O column. Trachea of mice was exposed and cannula was inserted into trachea and firmly tied with thread. Mice were ventilated at a speed of 150 breath/ min. Airway resistance of mice were recorded for six increasing doses of methacholine (2, 4, 8, 12, 16 and 20 mM) and normalized with basal level response of PBS.

4.2.3. Sample collection

Mice were euthanized and visceral organs were exposed to collect samples. Blood was collected, sera separated from blood by allowing it to clot following centrifugation at 400xg for 10 min at 4 ºC and kept at -20 ºC for immunoglobulin analysis. Bronchoalveolar lavage fluid (BALF) was collected by instillation of cold PBS into the lung. Approximately 1.2 ml of BALF was collected and centrifuged at 400 g for 10 min at 4 ºC. BALF supernatant was separated and preserved at -80 ºC for cytokine analysis. Lung was excised from thoracic cavity and fixed in neutral-buffered formalin for histology. Another part of lung was collected in PBS and kept at -80 ºC for analysis of kinase activation.

4.2.4. Western blot

Lungs homogenate were prepared in buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 100 µM sodium vanadate, 1 mM dithiothreitol, 5 mg/ml leupeptin, 1 mM phenylmethysulfonyl fluoride and centrifuged at 15,000g at 4ºC for 15 min (Kolliputi & Waxman, 2009). Supernatant was separated and protein content of lung homogenate (Hoarau et al., 2008) was determined by bicinchoninic acid assay. Lung homogenate was resolved in 12% SDS-PAGE. Protein from the gel was transferred to nitrocellulose membrane at 4ºC. Membrane was blocked with 3% bovine serum albumin in TBS (Tris-Buffered Saline), washed twice with TBST (Tris-Buffered Saline; 0.1% Tween-20) (10 min each in gentle shaking) and incubated with
primary antibody of phosphorylated Akt (Abcam, Cambridge, UK), ERK1/2 (Abcam, Cambridge, UK) and p38 (Santacruz Biotechnology, Texas, USA) in TBS for overnight at 4 °C. Membrane was washed twice with TBST (10 min each in gentle shaking), incubated with Anti-rabbit IgG-HRP, Anti mouse IgG1-HRP and Anti-rabbit IgG-HRP secondary antibody respectively at room temperature for 1h. Membrane was washed three times with TBST (10 min each in gentle shaking) and devolved using 50mM Sodium acetate, 15mg 3, 3’-Diaminobenzidine and 15 µl hydrogen peroxide (pH-5.0). Reaction was stopped using distilled water.

Densitometric analysis of blot was performed using “Image J” software. The intensity of obtained band was normalized with the band intensity of loading control and relative activation of kinase was plotted.

4.2.5. Cell count in BALF

The bronchoalveolar lavage cell pellet was resuspended in 100 µl PBS. Ten microliter of cell suspension was taken to enumerate total cell in BALF using trypan blue (Sigma Aldrich Co., St. Luis, USA) hemocytometer (Neuber’s chamber) and light microscope. BAL cell smear was prepared using grease free slides. Slides were stained with Leishman’s stain. Percentage of eosinophils and neutrophils was determined by counting minimum of 200 cells under microscope. Absolute numbers of eosinophils and neutrophils in per ml of BALF were determined by total cell count and percentage count (Mehta et al., 2007).

4.2.6. Immunoglobulins in sera

Ovalbumin specific IgE and IgG1 were measured in serum samples of mice by ELISA (Mehta et al., 2007). Briefly, 100 ng ovalbumin/well in 100µl of 0.1 M carbonate buffer (pH 9.6) were coated in microtiter plates (Nunc-Immuno, Denmark) by incubating the plate at 4°C overnight. Plates were washed two times with PBS and blocked with 3% defatted milk for 3 h at 37°C. Plates were washed two times with PBST (0.05% Tween-20 in PBS) followed by two times with PBS. Sera samples were diluted in PBS 1:10 and 1:50 (v/v) for IgE and IgG1 respectively and used in triplicates for estimation. Hundred microliters of diluted sera was added into the plates and incubated overnight at
4 °C. Unbound antibodies were removed by washing the plates three times with PBST followed by two times with PBS. For IgG1 plate was incubated at 37 °C for 3 h with anti-mouse IgG1-peroxidase (1:1000 PBS; BD Pharmingen, San Diego, CA, USA). IgE was estimated by incubating the plate with biotinylated anti-mouse IgE (2 µg/mL, BD Pharmingen) at 25°C for 90 min followed by washing two times with PBST followed by one time PBS and incubating again with streptavidin-peroxidase (1:1000; BD Pharmingen) for 30 min. After washing with PBST five times and two times with PBS, plates were developed using 0.1% ortho-phenylenediamine (w/v in buffer containing 14.4 M citric acid, 4.5 M Na2HPO4 and 0.001% H2O2 (30%)) and absorbance was read at 492 nm using microplate reader.

4.2.7. Estimation of cytokines in BALF

IL-4, IL-5, IL-10, IL-12 and IL-13 (eBioscience CA, USA) were determined in BALF by ELISA as per as manufacturer’s protocol. Briefly, 100 µl capture antibody (1:250 v/v) for each cytokine was coated separately in microtiter plates with carbonate buffer (pH 9.6). Plates were kept at 4°C overnight. After washing three times with PBST, plates were incubated with 10% fetal bovine serum (assay diluent) for blocking at 25°C for 1 hour. Seven serial dilutions of standards (7.8-500 pg/ml) were prepared in assay diluent and 100 µl of each standard were added to the wells in duplicates. BALF samples added into the plates and incubated for 2 h at 25°C and washed with PBS. After washing with PBST five times, plate were incubated with 100 µl (1:250 v/v in assay diluent) biotinylated detector antibody with avidin-horse radish peroxidise (HRP) at 25°C for 1 hour. To develop the plates, 100 µl of tetramethylbenzidine (Sigma Aldrich) substrate solution was added in and incubated at 37°C in dark for 30 min. Reaction was stopped by addition of 4N H2SO4 and absorbance was measured at 450 nm (wavelength correction at 570 nm) using microplate reader.

4.2.8. Histology

The lungs were fixed in 10% neutral buffered formalin (pH 6.8) and embedded in paraffin. Embedded lungs were cut into sections of 4 µm thickness. Lung sections were taken in glass slide and deparaffinized and hydrated (passed through xylene and ethanol
(100 to 0% in water)). Lung sections were stained with haematoxylin (0.5% w/v) followed by eosin (0.1% w/v). Slides were observed under light microscope and on the basis of cellular infiltration in peri-bronchial space, inflammation score was determined by experimentally blinded person and plotted in bar graph (in 1-10 scale).

4.2.9. Statistical analysis

The numerical values of each parameter were fed into the GraphPad Prism software (GraphPad Software, San Diego, CA, USA) in group wise manner. The experimental groups were compared for statistical difference using one way ANOVA (Analysis of variance) non-parametric test followed by Dunnett’s multiple comparison tests. Average value of each parameter was plotted in graph using Microsoft excel with SEM (Standard Error Mean).

4.3. RESULTS

4.3.1. Kinase inhibitor and AEBSF reduced airway resistance in mice

CE immunization to mice enhanced the methacholine induced airway resistance in comparison to control group (Figure 4.1). AEBSF given to mice significantly reduced airway resistant. Kinase inhibitor PI3K and ERK1/2 could also reduced the airway resistance significantly in mice (p<0.05). p38 could not reduce the CE induced airway resistance significantly. Combination of AEBSF along with PI3K, ERK1/2 reduced the airway resistance in mice (p<0.05).

4.3.2. Inhibition of ERK1/2, PI3k and Akt kinase in lung of mice

Western blot analysis revealed that the CE immunization to mice enhanced the activation of Akt, ERK1/2 and p38 in lung (Figure 4.2 & 4.3). Akt works downstream of PI3K hence activation of Akt was regarded as the activation of PI3K (Lee et al., 2006a). AEBSF monotherapy reduced the CE induced activation of Akt, ERK1/2 and p38 kinase. Mice those were given PI3K, ERK1/2 and p38 alone reduced the activation of corresponding kinase in the lung tissue. Combination of AEBSF with PI3K, ERK1/2 and the p38 reduced the activation of corresponding kinase in the lung tissue.
Figure 4.1: Airway resistances of mice in response to increasing doses of methacholine. Data represent mean ± SEM. *: p<0.05 as compared to CE immunized mice.

Figure 4.1: Airway resistances of mice in response to increasing doses of methacholine. Data represent mean ± SEM. *: p<0.05 as compared to CE immunized mice.
Figure 4.2: Western blot showing activation of kinases in lung homogenate of mice.
Figure 4.3: Relative level of phosphorylated kinase in lung of mice. Data represent mean ± SEM. *: p<0.05 as compared to CE immunized mice.
4.3.3. iPI3K and iERK1/2 potentiate AEBSF effect to reduce cellular infiltration

CE immunization to mice induced cellular infiltration into lung as found in the BALF. AEBSF given to CE immunized mice significantly reduced cell count in BALF. Among kinase inhibitor only iPI3K could significantly reduce the cell count in BALF (p< 0.05) (Figure 4.4). All treatment groups showed significantly reduced cell count except ip38 in BALF. Combination of AEBSF with iPI3Kand iERK1/2 showed more reduction in cell count (p< 0.001). The combination of iERK1/2 with AEBSF significantly reduced the cellular infiltration into lungs in comparison to iERK1/2 (p< 0.05).

CE induced eosinophil extravasation into the lung which was significantly reduced on AEBSF administration to mice (p< 0.05) (Figure 4.5). iPI3K and iERK1/2 (p< 0.05) was also effectively reduced eosinophil percentage in BALF. ip38 could not reduce the eosinophil infiltration in lung. The combination of AEBSF with iPI3K and with iERK1/2 showed significant reduction in eosinophil infiltration in lung (p< 0.001). The combination of AEBSF with iERK1/2 further reduced eosinophils percentage in lungs (p< 0.05), whereas combination of AEBSF with iPI3K significantly reduced the eosinophil in comparison to iPI3K (p< 0.05).

Mice immunized with CE showed increased extravasation of neutrophils in lungs (Figure 4.6). All the treatment given to mice significant reduced neutrophil count in BALF (p< 0.05) except ip38, iERK1/2 and combination of ip38 with AEBSF.

4.3.4. Inhibition of PI3K and ERK1/2 enhanced effect of AEBSF treatment for Th2 cytokine reduction

CE induced IL-4 secretion in mice as compared to the PBS control (Figure 4.2). Treatment of CE immunized mice with AEBSF reduced the IL-4 level in BALF (p< 0.05). Kinase inhibitor iPI3K and iERK1/2 given to mice also showed significant reduction in IL-4 level. Combination treatment of AEBSF with iPI3K and the iERK1/2 effectively reduced the IL-4 (p< 0.001) which was significantly lower than the monotherapy of iPI3K, iERK1/2 and AEBSF (p< 0.05).
Figure 4.4: Total cell count in BALF from different groups of mice. Data represent mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 as compared to CE immunized mice. #: p<0.05 as compared to AEBSF treated mice.
Figure 4.5: Eosinophil percentage in BALF. Data represent mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 as compared to CE immunized mice.
Figure 4.6: Neutrophil percentage in BALF. Data represent mean ± SEM. *: p<0.05; **: p<0.01 as compared to CE immunized mice.
CE immunized mice showed higher level of IL-5 in BALF, which was reduced significantly on AEBSF administration to mice (Figure 4.8). Kinase inhibitor $i$PI3K and $i$ERK1/2 except $i$p38 given to mice significantly reduced IL-5 in BALF ($p<0.05$). Maximum reduction in IL-5 level was observed in mice given combination of AEBSF with $i$PI3K and $i$ERK1/2.

Mice immunized with CE showed increased level of IL-13 in BALF (Figure 4.9). AEBSF administration to mice significantly reduced IL-13 level. Mice given, $i$PI3K and the $i$ERK1/2 also had reduced IL-13 level ($p<0.05$). Combination of AEBSF with $i$PI3K and the $i$ERK1/2 further augmented the IL-13 reduction ($p<0.001$) in comparison to monotherapy ($p<0.05$).

### 4.3.5. Serine protease inhibitor enhanced IL-10 cytokine

AEBSF significantly enhanced IL-10 level in CE immunized mice (Figure 4.10). However kinase inhibitor $i$PI3K, $i$ERK1/2 and $i$p38 administered to mice either alone or in combination with AEBSF could not enhanced the IL-10 level. The data indicate that kinase inhibitor could not induce the Treg population.

### 4.3.6. Combination of AEBSF with PI3K and the ERK1/2 inhibitor augmented IL-12 levels

AEBSF administered to CE immunized mice significantly increased the IL-12 level. However, none of the kinase inhibitor $i$PI3K, $i$ERK1/2 and $i$p38 could significantly increase the IL-12 as compared to CE immunized mice (Figure 4.11). Combination of AEBSF with $i$PI3K and $i$ERK1/2 further augmented the IL-12 level in CE immunized mice as compared to monotherapy with kinase inhibitor ($p<0.05$).
Figure 4.7: IL-4 levels in BALF measured by ELISA. Data represent mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 as compared to CE immunized mice. #: p<0.05 as compared to AEBSF treated mice.
**Figure 4.8:** IL-5 levels in BALF measured by ELISA. Data represent mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 as compared to CE immunized mice. #: p<0.05 as compared to AEBSF treated mice.
Figure 4.9: IL-13 levels in BALF measured by ELISA. Data represent mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 as compared to CE immunized mice. #: p<0.05 as compared to AEBSF treated mice.
**Figure 4.10:** IL-10 levels in BALF measured by ELISA. Data represent mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 as compared to CE immunized mice.
Figure 4.11: IL-12 levels in BALF measured by ELISA. Data represent mean ± SEM. 
*: p<0.05; **: p<0.01; ***: p<0.001 as compared to CE immunized mice. #: p<0.05 as compared to AEBSF treated mice.
4.3.7. Inhibitor of PI3K kinase augmented the effect of AEBSF to lowers immunoglobulin

CE immunization to mice induced IgE level. The serum IgE level was lower in mice those were given AEBSF (p< 0.05). None of the kinase inhibitor iPI3K, iERK1/2 and ip38 could reduce IgE significantly. Combination of AEBSF (p< 0.05) with iPI3K and the ip38 (p< 0.01) significantly reduced IgE (Figure 4.12a).

Mice immunized with CE showed enhanced serum IgG1 level (Figure 4.12b). AEBSF administration to mice significantly reduced IgG1 in mice. iPI3K was also effective in reduction of IgG1 in mice. Maximum reduction in IgG1 was observed in combination treatment of AEBSF with iPI3K (p < 0.001). Significant (p < 0.05) difference in IgG1 was observed when AEBSF was given in combination with iPI3K as compared to monotherapy.

4.3.8. Histological analysis of lungs

H&E stained lungs section of mice reveled that CE induces cellular infiltration in peri-bronchial and perivascular spaces in comparison to PBS control (Figure 4.13). AEBSF administration to CE immunized mice reduced the cellular infiltration. iPI3K, iERK1/2 also showed reduction in cellular infiltration in lung. Combination of AEBSF with iPI3K and the iERK1/2 also showed significant reduction in the cellular infiltration. Cellular infiltration in lung was further evaluated in terms of inflammation score (Figure 4.13j).
Figure 4.12: a) IgE and b) IgG1 in serum of mice. Data represent mean ± SEM. *: p<0.05; **: p<0.01; ***: p<0.001 as compared to CE immunized mice. #: p<0.05 as compared to AEBSF treated mice.
Figure 4.13: H&E stained Lung sections and inflammation score a) PBS, b) CE, c) AEBSF, d) iPI3K, e) iERK1/2, f) iP38, g) iPI3K+AEBSF, h) iERK1/2+AEBSF and i) iP38+AEBSF showing the cellular infiltration in different groups of mice. j) Inflammation score. The data represents the airway resistance mean ±SEM of six mice in each group (*p<0.05, **p<0.01, ***p<0.001).
4.4. DISCUSSION

Asthma is a disease of multifunction etiology. Prevalence of asthma and allergic disease has increased and become a major public-health concern (Devereux, 2006). Presently, corticosteroids, short and long-acting β2- adrenoceptor, leukotriene receptor antagonist, phosphodiesterase inhibitors, and mast cell stabilizers are existing therapies to take care the disease symptoms. The use of corticosteroids and β2- adrenoceptor agonists provides symptomatic relief, however long term use is associated with adverse effects (Roland et al., 2004; Dahl, 2006; Aaronson, 2006). PI3K, ERK1/2 and p38 are important kinases and activate to express inflammatory mediators in allergic diseases. These kinases can be target for the management of allergic disorders. Besides, protease inhibitors are also considered as potential therapeutic option which blocks the protease activity to reduce the release of inflammatory mediators (Clark et al., 1995; Elrod et al., 1997). A combination therapy has multiple advantages over the monotherapy (Peterlin et al., 2008; Straube et al., 2011) and may have additive or synergistic effect. Therefore, in the present study, the therapeutic effect of serine protease inhibitor in combination with PI3K, ERK1/2 and p38 kinase inhibitor were evaluated in CE induced mice model of allergic disease.

CE immunization to mice activated PI3K, ERK1/2 and p38 kinases in lung tissue and induced inflammatory mediators. Western blot confirmed that kinase inhibitors alone or in combination with AEBSF lowered the activation of corresponding kinase. Our data showed that the combination of (i)PI3K and (i)ERK1/2 with AEBSF reduced total cell and eosinophils in lungs. AEBSF, (i)ERK1/2 and the (i)PI3K reduced the allergen induced cellular and eosinophil infiltration in lungs of mice (Chialda et al., 2005; Duan et al., 2004; Duan et al., 2005; Saw & Arora, 2014).

The combination of AEBSF with (i)PI3K and the (i)ERK1/2 reduced Th2 cytokine significantly in comparison to individual monotherapy in mice. Chialda et al. demonstrated that the treatment of (i)PI3K reduced the ovalbumin induced immune response including Th2 cytokine followed by (i)ERK1/2, whereas (i)p38 treatment could not reduce the same. Reduction of Th2 cytokine by (i)PI3K and the (i)ERK1/2 are in agreement with earlier studies (Kane & Weiss, 2003; Pahl et al., 2002). PI3K showed involvement to augment T cell response by co-stimulation via CD28 following T cells
receptors engagement (Kane & Weiss, 2003). The \( \text{iP38} \) could not reduce Th2 cytokine in our study and also shown by others (Pahl et al., 2002; van den et al., 2001).

IL-12 cytokine is known to suppress Th2 induction (Gately et al., 1998). AEBSF given to mice enhanced IL-12 level in BALF. Combination of AEBSF with \( \text{iPI3K} \) and the \( \text{iERK1/2} \) increased IL-12 level and reduced Th2 cytokine more effectively than monotherapy. The better therapeutic effect of combination as comparison to monotherapy in lowering Th2 cytokine may attribute to increased IL-12 level. Our result are in agreement with Chen et al., (2006) that serine protease inhibitor can increase the IL-12 in mice. The effect of ERK1/2 and the PI3K inhibitor in IL-12 induction was demonstrated by in DC culture (Hoarau et al., 2008). PI3K act as endogenous inhibitor of IL-12 synthesis and suppress Th1 polarization (Fukao et al., 2002).

AEBSF given to mice in combination with \( \text{iPI3K} \) and the \( \text{ip38} \) showed effective reduction in serum IgE level. Pronounced reduction in IgE and IgG1 was observed in the combination treatment of AEBSF with \( \text{iPI3K} \) and the ip38. Earlier in vitro study showed that ip38 could directly reduce the IgE synthesis from human B cells by suppressing production of Ig epsilon germline transcripts, but neither \( \text{iPI3K} \) nor \( \text{iERK1/2} \). Same study also demonstrated that ip38 mediated reduction in IgE was not due to inhibition of B- cell proliferation (Zhang et al., 2002). Although in our study we did not get significant reduction in IgE for ip38 or iPI3K but maximum reduction was observed in IgG1 and IgE when mice were given ip38 or iPI3K in combination with AEBSF.

The existing pharmacotherapies for allergic diseases provide only symptomatic relief and require regular use. However, use in large doses over the long term is associated with adverse effects and development of drug resistance (Cockcroft & Swystun, 1996). Corticosteroids and \( \beta \)-adrenoreceptor agonists are mostly used in pharmacotherapy but the uses are implicated with insulin resistance, osteoporosis and cardiomyopathies (Ferris & Kahn, 2012; Waldeck, 2002). Hence, newer therapies are required to manage the allergic airway diseases. Previous studies have shown that serine protease inhibitor and the PI3K inhibitor are potential therapeutic agent could reduce
the allergic airway parameters in animal model. Our results suggest that combination of these two provide additive therapeutic advantage over monotherapy in Th2 mediated response.

In conclusion, PI3K inhibitor had best therapeutic effect with serine protease inhibitor to attenuate allergic response and has potential to be used as adjunct therapy in allergic airway diseases.