Summary and Conclusions
Prevalence of allergic diseases such as rhinitis, eczema, conjunctivitis, food allergy, urticaria etc. is increasing rapidly. Allergy is also a common cause for asthma. Asthma is an airway inflammatory disease characterized by chronic eosinophilic infiltration, bronchial responsiveness and variable airflow obstruction (Tattersfield et al., 2002). Antihistamines, anticholinergic agents, intranasal corticosteroids, mast cell stabilizers, bronchodilators like β-agonists, leukotriene inhibitors are being used for management of allergic diseases. However, these therapies provide only symptomatic relief and require regular use. Long term use of these drugs is associated with drug resistance and adverse effects; hence there is a need of an alternative therapy (Ariano et al., 2006).

During allergic reaction a number of inflammatory mediators are released from the immune cells which include histamines, cytokines, chemokines, leukotrienes and proteases. Serine proteases such as mast cell tryptase, chymase and elastase are important factor in allergic diseases which exaggerate allergic response in different ways. Tryptase facilitates histamine mediated allergic response such as bronchoconstriction and cellular infiltration (Molinari et al., 1996). Chymase and elastase participate in cellular infiltration and tissue remodeling (Frank et al., 2001). As serine proteases are implicated in pathophysiology of allergic airway diseases these inhibitors may have therapeutic promises. However, few of them are being studied, hence therapeutic evaluation and exploration of the new inhibitors is required.

AEBSF is an irreversible serine protease inhibitor with broad specificity and high affinity. It has low toxicity (LD$_{50}$ of 76mg/kg), excellent stability and solubility in water (200mg/ml). These properties are making AEBSF an interesting molecule to be studied for therapy. Present study is therefore aimed to explore the therapeutic effects of AEBSF in allergic airway disease. The present study was undertaken with the following objectives:

1) To study the prophylactic and therapeutic effect of AEBSF in ovalbumin induced airway inflammatory disease.
2) To evaluate the prophylactic and therapeutic effect of AEBSF in cockroach allergen induced airway inflammatory disease.
3) To study the effect of protease inhibitor in combination with kinase inhibitor in airway inflammatory disease.
1. Prophylactic and therapeutic effect of AEBSF in ovalbumin induced airway inflammatory disease

In the present study, prophylactic and therapeutic effect of AEBSF was evaluated in ovalbumin induced mice model of allergic airway disease. Mice were sensitized with ovalbumin (100 µg) on day 0 and 14 through i.p injection and challenged with ovalbumin (2 µg) on day 25, 26 and 27. Mice were administered with AEBSF (2, 10 and 50 µg) one hour before or after ovalbumin challenge. AEBSF was administered to mice through intranasal route to maximize the local effect of inhibitors in airway of allergic mice. On day 29, mice were euthanized, to collect serum BALF and lung to analyzed immunological and inflammatory parameters.

Ovalbumin immunization to mice induced cellular infiltration and Th2 cytokines (IL-4, IL-5 and IL-13) in BALF along with increase in specific serum IgE and IgG1. AEBSF treatment at 10 and 50 µg to ovalbumin immunized mice significantly reduced cellular infiltration including eosinophils and neutrophils. AEBSF given to mice either before or after allergen challenge reduced ovalbumin specific serum IgE and IgG1. On AEBSF treatment, Th2 cytokines were decreased along with increased IL-10 levels in BALF of immunized mice, indicating that shifting of Th2 immune response to regulatory T cell response. Previously, similar effect of serine protease inhibitor was demonstrated in Der p 1 allergen induced mice model of allergic disease (Chen et. al., 2006). Mice treated with AEBSF showed lower cysteinyl leukotrienes level in BALF. AEBSF treatment to mice before challenge (10 µg) or after challenge (2 µg and 10 µg) could also reduce the oxidative stress marker 8-isoprostane in BALF. Lung histology showed AEBSF administration to mice suppressed ovalbumin induced lung inflammation and mucus plugging. In conclusion, AEBSF reduces allergic airway inflammation and has potential for adjunct therapy in allergic diseases.

2. Prophylactic and therapeutic effect of AEBSF in cockroach allergen induced airway inflammatory disease

Ovalbumin is a purified allergen; however, naturally allergens are complex mixtures. To be used as a therapy, therapeutic effects of AEBSF have to be determined against different allergens. In this study, effect of AEBSF was evaluated in cockroach extract.
(CE; possesses multiple allergens with proteases) and Per a 10 (purified protease allergen) induced mice model of airway inflammatory disease. Mice were sensitized with CE (10 µg) or Per a 10 (10 µg) on day 0, 7 and 14 through i.p and challenged with cognate allergen (5 µg) through i.n route. Mice were given AEBSF (10 µg or 30 µg) one hour before or after allergen challenge. Mice were euthanized to collect blood BALF and lung for analysis of allergic parameters.

Allergen immunization to mice increased the methacholine induced airway resistance. AEBSF administration given to mice before or after challenge, dose dependently reduced methacholine induced airway resistance. AEBSF given to mice before allergen challenge reduced the airway resistance more effectively than treatment given after challenge possibly because of mast cell stabilizing property of protease inhibitor (Heet al., 2004).

CE or Per a 10 immunization to mice increased inflammatory and immunological parameters such as cellular infiltration, Th2 cytokines, and mucus plugging in lungs along with allergen specific serum IgE, IgG1. The present study showed that AEBSF treatment effectively reduced cellular infiltration including eosinophil and neutrophil in lung of both CE and Per a 10 immunized mice. AEBSF administration could also lower the allergen specific IgE and IgG1 in allergen immunized mice. On AEBSF administration to mice IL-4 and IL-13 lowered more effectively when given after allergen challenge. Mice administered with AEBSF showed reduced lung inflammation mucus plugging in airway and mast cell accumulation on sub epithelial surface of airway. AEBSF treatment to mice before or after allergen challenge showed significant decrease in 8-isoprostane level in BALF (Ishizaki et al., 2008). Same treatment also lowered the level of ROS in BAL cell pellet indicated the effect of AEBSF in reduction of allergen induced oxidative stress. Significant reductions in NF-κB activation in lung tissue indicate the therapeutic potential of AEBSF in allergic airway diseases (Chen et al., 2006). In conclusion, AEBSF reduced the airway hyper responsiveness, inflammation and oxidative stress effectively in mouse model, independent of protease activity of allergen. Furthermore, AEBSF promises add-on therapy or combination therapy for allergic airway diseases.
3. Effect of protease inhibitor in combination with kinase inhibitor in airway inflammatory disease

PI3K, ERK1/2 and p38 kinase are important signaling molecule in the exacerbation of allergic diseases. Indeed, a complex interplay of these kinases are involved in immune cell activation and release of inflammatory mediators. Activation of PI3K and ERK1/2 kinase are involved in T cell activation proliferation and cytokine induction (Egerton et al., 2012). P38 kinase is involved in release of inflammatory mediators such as TNF-α, IL-1β (Schieven, 2005). Targeting kinases are emerging strategy for the management of allergic airway diseases. Besides, serine protease inhibitors are also considered as potential therapy. Both serine protease inhibitors and kinase inhibitors have therapeutic potential in allergic diseases, but combination may have better therapeutic potential. 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.

Mice were sensitized with CE (100 µg by i.p) on day 0, 7 and 14 and challenged with CE on day 27, 28 and 29 with CE (5 µg through i.n). Mice were administered with kinase inhibitors of PI3K (iPI3K; 3mg/kg), ERK1/2 (iERK1/2; 3mg/kg), p38 (ip38; 3mg/kg) or AEBSF (30 µg) through i.n one hour before challenge. Mice were also given combination of AEBSF with each of afore mentioned kinase inhibitors. Mice were euthanized to collect blood BALF and lung sample for further analysis.

CE immunization to mice activated PI3K, ERK1/2 and p38 kinase in lung tissue and induced inflammatory mediators. Western blot of lung homogenate confirmed that kinase inhibitors alone or in combination with AEBSF lowered the activation of corresponding kinase. Enhanced effect in reduction in cellular infiltration including eosinophils was observed on treatment of AEBSF in combination with iPI3K and iERK1/2 compared to monotherapy. Combination of AEBSF with iPI3K and the iERK1/2 augment the reduction of Th2 cytokine in comparison to monotherapy. The effect of iPI3K and iERK1/2 monotherapy to reduce Th2 response has already been demonstrated in allergic diseases (Kane & Weiss, 2003; Pahl et al., 2002). Combination of AEBSF with iPI3K and the iERK1/2 showed enhanced level of IL-12.
as compared to monotherapy. Previously, the role of \( i\text{PI3K} \) and the \( i\text{ERK1/2} \) in IL-12 induction was demonstrated in DC culture (Hoarau et al., 2008). The augmented effect of AEBSF to attenuate Th2 cytokine in combination with \( i\text{ERK1/2} \) and the \( i\text{PI3K} \) was probably attributed to increased level of IL-12. AEBSF given to mice in combination with \( i\text{PI3K} \) and the \( i\text{p38} \) showed effective reduction in serum IgE level. Pronounced reduction in IgE and IgG1 was observed in the combination treatment of AEBSF with \( i\text{PI3K} \) and the \( i\text{p38} \).

Combination of AEBSF with \( i\text{PI3K} \) was most effective in reduction of CE induced immune response in mouse. One reason for the best effect of \( i\text{PI3K} \) combination could be the upstream role of PI3K Kinase. In conclusion, PI3K inhibitor had best therapeutic effect with serine protease inhibitor to attenuate allergic diseases and the combination has additive therapeutic potential in allergic airway diseases.

**Highlights of the studies**

- Intranasal administration of AEBSF attenuated ovalbumin induced cellular infiltration, lung inflammation and mucus plugging.
- Serine protease inhibitor reduced ovalbumin induced IgE and IgG1 in mice.
- AEBSF administration reduced Th2 cytokine and increased IL-10 in allergen immunized mice indicating shift in immune response from Th2 to Treg.
- AEBSF treatment lowered ovalbumin induced CysteinyI leukotrienes in BALF of mice.
- Administration of AEBSF minimized ovalbumin induced oxidative stress in lung.
- Serine protease inhibitor treatment before allergen challenge was more effective in reduction of methacholine induced airway resistance.
- Intranasal administration of AEBSF in cockroach allergen immunized mice lowered cellular infiltration Th2 cytokines in lung and allergen specific IgE and IgG1 in serum.
- AEBSF lowered lung inflammation mucus plugging and mast cell accumulation in lung of cockroach allergen immunized mice.
Administration of AEBSF reduced cockroach allergen induced oxidative stress in mice.

AEBSF lowered NF-κB activation in lung, which is an important transcription factor for induction of inflammatory mediators.

Combination of PI3K and ERK1/2 inhibitor to serine protease inhibitor enhanced lowering of allergen induced cellular infiltration and lung inflammation.

PI3K and ERK1/2 inhibitor in combination with AEBSF augmented IL-12 level.

Combination of PI3K and ERK1/2 inhibitor with AEBSF augmented reduction in Th2 cytokines seems to be IL-12 mediated response.