Indian Journal of Experimental Biology
Vol. 53, October 2015, pp. 625-631

Cellular and molecular mechanisms of action of polyherbal preparation UNIM-352 in experimental models of bronchial asthma

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Bronchial asthma is a chronic inflammatory disorder of the airways and pharmacotherapy is dependent on anti-inflammatory and bronchodilator agents. However, adverse effects of these agents on chronic administration and sometimes non-responsiveness to these drugs have prompted the search for viable alternatives from medicinal plant sources. UNIM-352 is a polyherbal preparation traditionally used in the Unani system of Indian medicine for the treatment of bronchial asthma. The present study defines the possible cellular and molecular mechanisms of action of UNIM-352 in experimental models of bronchial asthma and validates the observed therapeutically beneficial effects. Wistar rats were immunized and challenged with ovalbumin, and blood and bronchoalveolar lavage (BAL) fluid were assayed for cytological and biochemical markers. UNIM-352 (200 and 400 mg/kg) markedly reduced the eosinophil and neutrophil counts in both blood and BAL compared to control. The polyherbal agent also attenuated the levels of TNF-α, IL-4, GM-CSF and NF-κB whereas histone deacetylase (HDAC) levels were elevated, in both blood and BAL fluid. All effects of UNIM-352 were comparable with the standard drug, prednisolone. The results demonstrated possible cellular and molecular mechanisms of UNIM-352 and thus explain its beneficial effects in bronchial asthma.

Keywords: Bronchodilators, Cytokines, Herbal, Histone deacetylase, Indian medicine, Inflammation, NF-κB, Prednisolone, Unani.

Bronchial asthma is a complex chronic inflammatory disease of the airways characterized by airway hyper-responsiveness, airflow obstruction and airway remodeling and regulated by cellular and humoral factors[1]. It involves recruitment and activation of many inflammatory and structural cells such as mast cells, macrophages, eosinophils, T-lymphocytes, neutrophils, epithelial and smooth muscle cells. Some of these cells are capable of synthesizing and releasing inflammatory mediators that are central to the pathophysiology of the disease[2-4]. For example, T-lymphocytes (particularly Th2 type) and mast cells produce various cytokines (IL-4, GM-CSF, TNF-α, etc.), which are crucial humoral players in the process[5-7]. Pharmacotherapy of bronchial asthma consists of anti-inflammatory agents (corticosteroids) and bronchodilators (β-2-agonists). However, significant incidence of adverse effects related to these drugs has been a major area of concern[8]. In addition, the increasing incidence of refractoriness to conventional forms of therapy has further complicated the problem[9]. As a result, search for newer, alternative and more viable therapeutic strategies for the control of bronchial asthma has started.

In traditional systems of medicine, the medicinal plants play a crucial role and constitute a major source for therapeutic agents. Focus on herbal drug research has been intensified all over the world since one decade and the immense potential of such herbal agents have been recognized[10-15]. UNIM-352 is a polyherbal Unani formulation shown to be effective in experimental model of bronchial asthma[16,17]. A pilot clinical study has shown that UNIM-352 can be used as an adjunct in the treatment of bronchial asthma as it increased the efficacy of conventional drug treatment by improving lung function and fewer incidences of acute exacerbations of the disease[18-20]. As inflammation leads to bronchial hyper-reactivity and reversible airway obstruction in bronchial asthma, here, we assessed the effects of the UNIM-352 on inflammatory markers in the bronchoalveolar lavage (BAL) fluid and blood. Also, we investigated the possible cellular and molecular mechanisms of action of UNIM-352 in experimental model of bronchial asthma (Ovalbumin sensitized and challenged). Prednisolone[21,22] was used as the comparator drug

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as it is clinically widely used in patients of bronchial asthma as controller medication for its anti-inflammatory effects.

Materials and Methods

Animals—Wistar rats of either sex (180-220 g) were used for the study. The animals were maintained under standard laboratory conditions of light:dark 12 h cycle and temperature (25±2°C) and had free access to food and water. The animal care was as per guidelines laid down by the Indian National Science Academy, New Delhi and the study protocol was approved by the Institutional Animal Ethics Committee (Reference no. IAEC/7/2011).

Drugs and Chemicals—The ingredients of polyherbal preparation UNIM-352 are known medicinal plants: *Linum usitatissimum*[23], *Trigonella foenum-graecum*[24], *Allium sativum*[25], *Strychnos potatorum*[26], *Caesalpinia bonducella*[27] and *Pongamia glabra*[28]. The drug was formulated (as an aqueous extract) and standardized by the Central Research Institute of Unani Medicine, Hyderabad. Ovalbumin (OVA) and Prednisolone were procured from Sigma-Aldrich, USA. The ELISA assay kits (rat) for TNF-α (Catalog: 865.000.096) and IL-4 (Catalog: 865.020.096) were procured from Diaclone, France, whereas assay kits for GM-CSF (Catalog: E0045r), NF-κB (Catalog: E1824r) and histone deacetylase (Catalog: E0883r) were procured from USCN life science and technology Co. Ltd., China. All other routine and standard laboratory reagents were procured from Sisco Research Labs, New Delhi.

Experimental procedure—Rats were divided into four groups (n=6 each) *viz*., (I) control [vehicle (distilled water)]; (II & III) UNIM-352 @ 200 and 400 mg/kg, respectively; and (IV) prednisolone @ 10 mg/kg; administered orally for 15 days. Gr IV treated with prednisolone served as the positive control. All groups were immunized with ovalbumin (10 mg/rat, i.p.) adsorbed to 10 µg of aluminium hydroxide. Fourteen days after immunization, the animals were challenged with ovalbumin (1 mg) in 0.5 ml of isotonic saline[29].

Blood and bronchoalveolar lavage (*BAL*) *fluid collection and cell counts*—After 24 h of ovalbumin challenge, rats were anesthetized and blood was collected by cardiac puncture. Blood was centrifuged at 4ºC (3000 rpm) for 10 min and the serum was separated and stored at −80ºC. BAL fluid was collected by lavaging the lung through a tracheal cannula with 0.9% sodium chloride solution and centrifuged at 1500 rpm at 4ºC for 10 min and supernatant recovered and stored at −80ºC for assay of various biochemical markers[30]. The precipitated pellets were resuspended in 100 µl of normal saline. Eosinophil and neutrophil counts in blood and BAL fluid were carried out using Neubauer chamber.

Assay for cytokines, *NF-κB and HDAC*—Blood and BAL fluid samples were assayed for levels of TNF-α, IL-4, GM-CSF, NF-κB and histone deacetylase (HDAC) using commercially available ELISA kits. Briefly, the cytokine assays were performed using the solid phase sandwich ELISA. Antigen and biotinylated polyclonal antibody specific for TNF-α, IL-4 or GM-CSF were added to the microtitre plate wells whose walls pre-coated with polyclonal antibody specific for rat TNF-α, IL-4 or GM-CSF and incubated for specified periods. The HRP conjugated streptoavidin was added and incubated. Further, TMB substrate solution was added to induce a coloured reaction product. The enzyme substrate reaction was stopped by adding H_2SO_4. The absorbance of the coloured product was measured using the software based microplate reader (ECIL) at 450 nm and results were expressed in pg/ml.

NF-κB and HDAC assays were performed using sandwich ELISA. The microtitre plate provided in this kit was pre-coated with an antibody specific to NF-κB and HDAC. Samples and biotin-conjugated polyclonal antibody specific for NF-κB and HDAC was added and incubated. Later, avidin conjugated to HRP was added and incubated. The HRP conjugated avidin was reacted with a chromogenic substrate reagent and reaction was stopped. The absorbance of the coloured product was measured using the software based microplate reader (ECIL) at 450 nm and results were expressed in ng/ml.

Statistical analysis—All data are expressed as mean ± SEM and analyzed by using one-way ANOVA followed by Dunnet`s test. A *P* value of $\geq$0.05 was used as the level of significance in all statistical tests.

Results

Effect of UNIM-352 on eosinophils and neutrophils in blood and BAL fluid—In this experiment, effects of traditional polyherbal Unani preparation UNIM-352 was assessed on eosinophil and neutrophil cell counts in blood and bronchoalveolar lavage (BAL) fluid in OVA immunized rats. UNIM-352 (200 and 400 mg/kg) reduced number of eosinophil cells, at

both the dose levels, by 40 and 74% in blood and by 59 and 81% in BAL, respectively when compared with vehicle treated control group. On the other hand, with prednisolone pre-treatment the number of eosinophil cells were more markedly suppressed by 91% in blood and 92% in BAL. Analysis of the data revealed that the changes in number of eosinophil cells after various treatments were significantly different across all groups [F (3, 23) = 6.1, for blood; and F (3, 23) = 6.2, for BAL; P <0.01 in each case]. These results are summarized in Fig. 1A.

Similarly, UNIM-352 (200 and 400 mg/kg) reduced number of neutrophil cells, at both the dose levels, by 25 and 40% in blood and by 28 and 46% in BAL, respectively compared to the vehicle treated control group. On the other hand, with prednisolone pre-treatment the number of neutrophil cells was markedly suppressed by 72% in blood and 73% in BAL. Analysis of the data revealed that the changes in number of neutrophil cells after various treatments were significantly different across all groups [F (3, 23) = 14.1, P <0.01 for blood; and F (3, 23) = 6.7, P <0.01 for BAL fluid]. These results are depicted in Fig. 1B.

Effect of UNIM-352 on cytokine levels—Assay for TNF-α showed that UNIM-352 (200 and 400 mg/kg) induced suppression in both blood and BAL fluidsin a dose dependent manner. There were 45 and 54% reductions of the cytokine levels in blood and 28 and 41% in BAL fluid, respectively as compared to controls. Whereas, prednisolone induced suppressions in TNF-α were 77% in blood and 61% in BAL. The changes in TNF-α levels after various treatments were significant across all groups [F (3, 23) = 8.5, for blood; and F (3, 23) = 13.6, for BAL fluid; P <0.01 in each case] (Fig. 1C).

Similarly, assay for IL-4 showed that at both the doses (200 and 400 mg/kg), UNIM-352 induced suppression of IL-4 levels by 30 and 60% in blood and by 29 and 43% in BAL fluid, respectively as compared to controls. Whereas, prednisolone induced suppressions were 75% in blood and 77% in BAL. The changes in IL-4 levels after various treatments were significant across all groups [F (3, 23) = 44.2, for blood; and F (3, 23) = 31.3, for BAL fluid; P <0.01 in each case] (Fig. 1D).

Assay for GM-CSF showed that at both lower and higher doses of UNIM-352 (200 and 400 mg/kg) the GM-CSF levels were suppressed by 16 and 20% in blood and by 15 and 25% in BAL, respectively as compared to controls. Whereas, prednisolone induced suppressions were 29% in blood and 33% in BAL. The changes in GM-CSF levels after these treatments were significant across all groups [F (3, 23) = 4.6, P <0.05 for blood; and F (3, 23) = 7.2, P <0.01 for BAL fluid] (Fig. 1E).

Effect of UNIM-352 on NF-κB and HDAC levels—Assay for NF-κB showed that both lower and higher doses of UNIM-352 (200 and 400 mg/kg) induced suppression of NF-κB levels by 2 and 3% in blood and by 41 and 60% in BAL, respectively as compared to controls. This suppression was comparable to that of prednisolone *i.e*., 3% in blood and 67% in BAL. The changes in NF-κB levels after various treatments were significant in case of BAL fluid [F (3, 23) = 4.1, P <0.05 for BAL fluid];

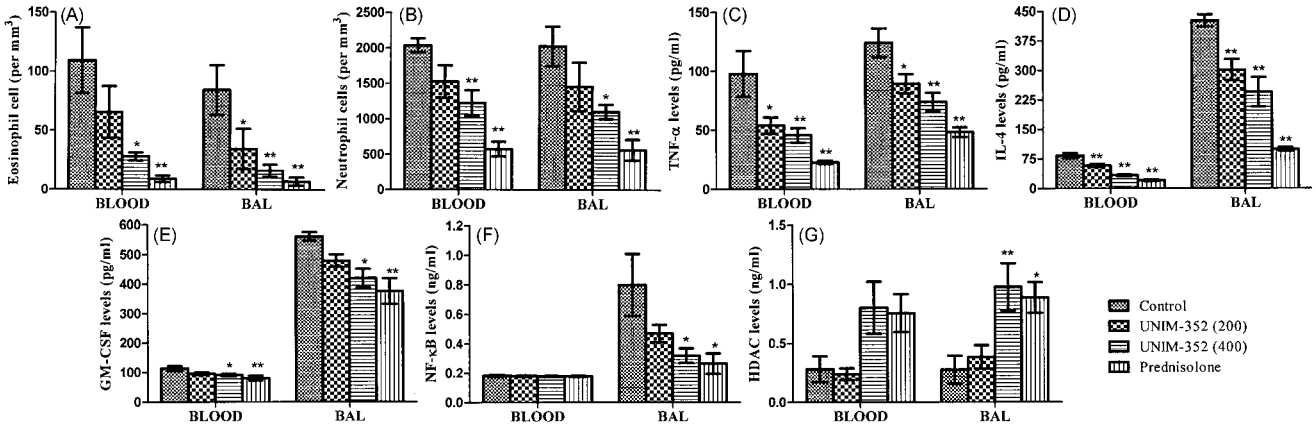

Fig. 1—Effect of polyherbal preparation UNIM-352 on (A) eosinophil counts; (B) neutrophil counts; (C) TNF-α; (D) IL-4; (E) GM-CSF; (F) NF-κB; (G) histone deacetylase (HDAC) levels in blood and BAL fluid in ovalbumin immunuized rats. [Control, OVA-sensitized and challenged rats treated with vehicle; UNIM-352 (200) & UNIM-352 (400), OVA-sensitized and challenged rats treated with UNIM-352 @ 200 and 400 mg/kg orally, respectively; Prednisolone, OVA-sensitized and challenged rats treated with Prednisolone @ 10 mg/kg. Data are expressed as Mean ± SEM. $^{*}P$ <0.05 and $^{**}P$ <0.01, as compared to control group.]

but not significant in case of blood [F (3, 23) = 0.2, P >0.05 NS for blood] (Fig. 1F).

Assay for HDAC also exhibited suppression by 15% in blood at lower dose of UNIM-352 (200 mg/kg). However, HDAC levels in BAL were elevated by 39%. At higher dose of UNIM-352 (400 mg/kg), HDAC levels showed elevation by 185% in blood and 254% in BAL as compared to controls. Prednisolone induced elevation was 169% in blood and 221% in BAL. These changes in HDAC levels after various treatments were significant across all groups [F (3, 23) = 4.1, P <0.05 for blood; and F (3, 23) = 6.9, P <0.01 for BAL] (Fig. 1G).

Discussion

Complex interactions between cellular and humoral components play a crucial role in the regulation of pathophysiology of bronchial asthma. Pharmacotherapy of this condition depends on controller (corticosteroids) and reliever (bronchodilators) agents. Management of bronchial asthma requires long term treatment with both the groups of agents and has given rise to issues like reduced efficacy and safety concerns. In this context, search for safer and effective alternative strategies for rational management of the disorder has turned the attention of researcher towards medicinal plants and their derived products. Though, information on such sources are available in traditional system of Indian medicine, there is need for scientific validation of such claims before recommendation for complimentary therapy.

UNIM-352 is one such polyherbal Unani preparation with documented clinical use in patients of bronchial asthma in traditional medicine[18-20]. Our pilot clinical study has shown that UNIM-352 improves the efficacy and safety of conventional treatment of bronchial asthma in modern medicine and thus, could be considered as a viable adjunct for pharmacotherapy[18].

Reverse pharmacology is the science of integrating documented clinical/experiential hits, into leads by trans-disciplinary exploratory studies and further developing these into drug candidates by experimental and/or clinical research. It is an alternative mode of drug development with special relevance to medicinal plants and is aimed at understanding the mechanisms of action at multiple levels of biological organization with a view to optimize safety, efficacy and acceptability of the leads in natural products, based on relevant science. In our present study, we adopted this reverse pharmacology approach to experimentally evaluate the possible cellular and molecular mechanisms involved in the effects of UNIM-352 in the rat model of bronchial asthma.

The inflammatory process in asthma involves antigen interaction and sustained infiltration and activation of many inflammatory cells including lymphocytes, eosinophils, neutrophils, basophils and macrophages, followed by synthesis and release of various pro-inflammatory mediators and cytokines[31-33]. It is well described that Th2 cells are the key orchestrators of this inflammation, initiating and propagating inflammation through the release of cytokines, TNF-α, IL-4, GM-CSF in turn recruiting and activating eosinophils and neutrophils, the effector cells in asthma[34-36]. TNF-α, a well known pro-inflammatory mediator, is a potent modulator of immune and inflammatory responses. It causes an influx of neutrophils and eosinophils as well as bronchial hyperreactivity[37]. IL-4 induces IgE isotype switching in B-lymphocytes and mucus production by goblet cells, as well as upregulation of the expression of adhesion molecules required for inflammatory cell recruitment[30]. GM-CSF is also a key cytokine, that not only activates eosinophils and prolongs eosinophil survival in the peripheral tissues but also influences the growth and differentiation of antigen-presenting cells (APC)[38].

Cellular inflammation of the airways with eosinophils and neutrophils is a characteristic feature of asthma and is considered relevant to the pathogenesis of the disease. In most asthma phenotypes, increased levels of eosinophils and neutrophils in the tissues, blood and bronchoalveolar lavage (BAL) fluid and in general, is correlated with the disease severity[39]. The results of our experiments showed that treatment with the polyherbal preparation, UNIM-352, at both the dose levels (200 and 400 mg/kg) reduced the number of eosinophil and neutrophil cells presented in both blood and BAL fluid of ovalbumin immunized and challenged rats. Prednisolone (10 mg/kg) treatment, which acted as a positive control group, also decreased the number of eosinophil and neutrophil cells in a separate group of rats indicating the comparability of our data.

TNF-α is released in allergic responses from both mast cells and macrophages via IgE-dependent mechanisms, and elevated levels have been demonstrated in the BAL fluid of asthmatic subjects undergoing allergen challenge. The acute asthmatic

response to allergen is mediated by sensitized mast cells whose high affinity IgE receptors (FcεRI receptors) are occupied by IgE directed against specific allergens. Allergen crosslinks IgE molecules attached to mast cell surface, causing degranulation. Mast cells have been shown to generate a range of mediators including cytokines. It indicates that the cytokines are co-released with the more extensively characterized preformed mast cell granule mediators, such as histamine, chymase and tryptase. Mast cell mediators are classically associated with immediate bronchospasm, and now TNF-α has also been shown to induce airway hyperreactivity[40]. Our results showed that the levels of TNF-α were significantly reduced in both blood and BAL fluid in ovalbumin sensitized and challenged rats after UNIM-352 treatment suggesting its ability to lower levels of this crucial pro-inflammatory cytokine. IL-4 acts on B cells to facilitate IgE production and on mast cells leading to their activation. This activation of mast cells and the subsequent release of cytokines cause acute symptoms of asthma, such as sneezing and bronchospasm[41]. Our results showed significantly reduced levels of IL-4 after UNIM-352 treatment in both blood and BAL fluid in ovalbumin sensitized and challenged rats. Since IL-4 is a Th2 dependent cytokine and a marker for the activity of this lymphocyte subset which plays a key role in allergic responses, UNIM-352 could be beneficial in asthma of allergic origin.

GM-CSF plays an important function in the growth, development, and maturation of granulocytes, macrophages, and dendritic cells. Although GM-CSF was originally considered as a hemopoietic cytokine, more recent studies indicate that it also has a major role in a variety of inflammatory responses. GM-CSF has important effects on eosinophils which are prominent in allergic inflammation. Not only it accelerates the growth and maturation of eosinophils but also primes them for activation and enhances their survival[42]. The results of our experiments showed that treatment with the polyherbal preparation, UNIM-352, at both the dose levels (200 and 400 mg/kg, orally), reduced the level of GM-CSF in both blood and BAL fluid as compared to control group rats. These results suggest an anti-inflammatory mode of action for the polyherbal agent.

Inflammatory lung diseases are characterized by increased expression of many inflammatory genes that are regulated by pro-inflammatory transcription factors, such as NF-κB. It is normally present in the cytoplasm in an inactive form associated to an inhibitory protein IκB. Exposure to allergen, viruses, bacteria and pro-oxidants increase the release of pro-inflammatory cytokines (TNF-α, IL-1β) from macrophages and other inflammatory cells. Further, IκB proteins are phosphorylated and degraded which results in a rapid translocation of NF-κB to the nucleus and transcriptional initiation of NF-κB dependent genes encoding inflammatory proteins such as TNF-α, GM-CSF[43,44]. The results of our experiments have demonstrated that treatment with the polyherbal preparation UNIM-352, at both the dose levels (200 and 400 mg/kg), and prednisolone reduced the level of NF-κB in BAL fluid as compared to control group rats. Expression of NF-κB also results in activation of histone acetyltransferase (HAT) leading to histone acetylation. Histone acetylation plays a critical role in the regulation of inflammatory genes expression. Histone deacetylase (HDAC) plays an important role in the inhibition of gene expression by reversing the hyper acetylation of histones. In bronchial asthma, there is a marked increase in HAT and reduction in HDAC activity[45,46]. Our results showed that the levels of HDAC were increased in both blood and BAL fluid in ovalbumin sensitized and challenged rats after UNIM-352 treatment which was comparable to what was observed in prednisolone treated group. It can be suggested that UNIM-352 may reverse the histone acetylation by recruiting histone deacetylase and thereby switch off the inflammatory genes, and this may explain the observed beneficial effects of UNIM-352 in patients of bronchial asthma.

Taken together, it can be inferred that the polyherbal preparation UNIM-352 acts by preventing infiltration of the eosinophils and neutrophils as well as by inhibiting the activity of pro-inflammatory and immunomodulatory cytokines such as TNF-α, IL-4, GM-CSF at the site of inflammation. Further, it also blocks the inflammatory gene transcription by inhibition of NF-κB and activation of histone deacetylase. These cellular and molecular mechanisms of action may explain its observed beneficial effects in the experimental model of bronchial asthma.

Acknowledgements

The financial support from the Department of AYUSH, Government of India, New Delhi is gratefully acknowledged. The authors thank Central Council for Research in Unani Medicine (CCRUM) for supply of UNIM-352.

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