



## Implication of novel thiazolo-thiophene derivative (MCD-KV-10) for management of asthma

Dhiraj Patil, Ranjeet Prasad Dash, Sandeep Kumar Thakur, Amit N. Pandya, P. Venkatesh, Kamala K. Vasu & Manish Nivsarkar

**To cite this article:** Dhiraj Patil, Ranjeet Prasad Dash, Sandeep Kumar Thakur, Amit N. Pandya, P. Venkatesh, Kamala K. Vasu & Manish Nivsarkar (2015) Implication of novel thiazolo-thiophene derivative (MCD-KV-10) for management of asthma, Journal of Enzyme Inhibition and Medicinal Chemistry, 30:2, 229-239, DOI: [10.3109/14756366.2014.913035](https://doi.org/10.3109/14756366.2014.913035)

**To link to this article:** <https://doi.org/10.3109/14756366.2014.913035>

 View supplementary material [↗](#)

 Published online: 18 Jun 2014.

 Submit your article to this journal [↗](#)

 Article views: 811

 View related articles [↗](#)

 View Crossmark data [↗](#)

ORIGINAL ARTICLE

## Implication of novel thiazolo-thiophene derivative (MCD-KV-10) for management of asthma\*

Dhiraj Patil<sup>1</sup>, Ranjeet Prasad Dash<sup>2</sup>, Sandeep Kumar Thakur<sup>2</sup>, Amit N. Pandya<sup>3</sup>, P. Venkatesh<sup>1</sup>, Kamala K. Vasu<sup>3</sup>, and Manish Nivsarkar<sup>2</sup>

<sup>1</sup>Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Education and Research – Ahmedabad, C/O – B. V. Patel Pharmaceutical Education and Research Development (PERD) Centre, S. G. Highway, Thaltej, Ahmedabad, Gujarat, India, <sup>2</sup>Department of Pharmacology and Toxicology, and <sup>3</sup>Department of Medicinal Chemistry, B. V. Patel Pharmaceutical Education and Research Development (PERD) Centre, S. G. Highway, Thaltej, Ahmedabad, Gujarat, India

### Abstract

**Context:** Asthma is multifaceted disease where many targets contribute towards its development and progression. Among these, adenosine receptor subtypes play a major role.

**Objective:** MCD-KV-10, a novel thiazolo-thiophene was designed and evaluated pre-clinically for its implication in management of asthma.

**Materials and methods:** This compound showed good affinity and selectivity towards A<sub>2A</sub>/A<sub>3</sub> adenosine receptor (AR) subtypes. Furthermore, MCD-KV-10 was evaluated for *in vitro* lipoxygenase inhibition activity; *in vivo* mast cell stabilization potential and *in vivo* anti-asthmatic activity was done in ovalbumin-induced airway inflammation model in guinea pigs.

**Results:** The compound showed good (>57%) inhibition of lipoxygenase enzyme and also effectively protected mast cell degranulation (>63%). The compound showed good anti-asthmatic activity as inferred from the *in vivo* studies.

**Discussion:** These results indicate that MCD-KV-10 has an inhibitory effect on airway inflammation.

**Conclusion:** Though, we have identified a potential candidate for management of asthma, further mechanistic studies are needed.

### Keywords

Adenosine, asthma, cytokines, lipoxygenase, thiazolo-thiophene

### History

Received 29 September 2013

Revised 26 March 2014

Accepted 26 March 2014

Published online 17 June 2014

### Introduction

Adenosine receptors are G-protein-coupled receptors which mediate its biological action *via* its four subtypes, A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub>-adenosine receptors (AR)<sup>1</sup>. The biochemical interaction of adenosine with its receptors initiates signals transduction pathways, including the adenylyl cyclase effector system, utilizing cAMP as a second messenger. A decrease in intracellular levels of cAMP occurs due to coupling of A<sub>1</sub> and A<sub>3</sub> adenosine receptors (A<sub>1</sub>-AR, A<sub>3</sub>-AR) to G<sub>i</sub> proteins followed by inhibition of adenylyl cyclase. However, an upsurge of cAMP levels occurs upon coupling of A<sub>2A</sub>/A<sub>2B</sub> adenosine receptor to G<sub>s</sub> proteins leading to stimulation of adenylyl cyclase<sup>2</sup>. Studies have demonstrated the synergy between adenosine receptor activation and allergens in inducing mast cell degranulation<sup>3,4</sup>. The involvement of A<sub>1</sub>-ARs have been identified on a number of different human cell types that are important in the pathophysiology of asthma, including human airway epithelial and bronchial smooth muscle cells, lymphocytes, mast cells, neutrophils, monocytes,

macrophages, fibroblasts and endothelial cells<sup>5–19</sup>. Activation of A<sub>1</sub>-ARs subsequently induces the release of mediators and cytokines that lead to airway hyper-reactivity, inflammation and airway remodeling. It has been found that activation of A<sub>1</sub>-ARs on human asthmatic bronchial tissue produces bronchoconstriction<sup>20</sup>. A<sub>2A</sub> also play a major role in development and progression of asthma. The specific contribution of A<sub>2A</sub>-AR in relevance to asthma is: selective activation of the A<sub>2A</sub> receptor inhibits histamine and tryptase release from mast cells<sup>21–23</sup>, adherence of neutrophil to the endothelium<sup>6,24</sup>, integrin upregulation<sup>25</sup>, respiratory burst<sup>26,27</sup> and degranulation of mast cells<sup>28</sup>. Furthermore, the A<sub>2A</sub> receptor suppresses the activation and expansion of T-lymphocytes<sup>29</sup>. It has been predicted that a selective A<sub>2A</sub> receptor agonist may have broad-spectrum anti-inflammatory effects similar to glucocorticosteroids, by exerting inhibitory effects on multiple inflammatory cell types.

A<sub>3</sub>-ARs also play a crucial role in the progression and development of asthma. Activation of A<sub>3</sub>-ARs induces the release of basophils and produces bronchoconstriction, eosinophil migration into airways and mucus hypersecretion in animals. Thus, A<sub>3</sub>-AR antagonists have been suggested for development as anti-asthma drugs<sup>30,31</sup>.

Our efforts in developing novel ligands for AR subtypes resulted in the identification of 2-aminoimidazole as a privileged structure to achieve selectivity towards A<sub>2B</sub> AR subtype

\*NIPER-Ahmedabad Submission No – NIPERA250213.

Address for correspondence: Dr. Manish Nivsarkar, Director, B. V. Patel Pharmaceutical Education and Research Development (PERD) Centre, S. G. Highway, Thaltej, Ahmedabad 380054, Gujarat, India. Tel: +91 79 27413219. Fax: +91 79 27450449. E-mail: manishnivsarkar@gmail.com

(unpublished data). Along with this, we have also identified 2-amino-5-benzoyl-4-phenylthiazole as a potential AR<sub>1</sub>-AR receptor antagonist<sup>32</sup>. Based on our previous findings, a series of novel thiazole derivatives were synthesized with a view of exploring their potential as ligands for AR subtypes. Our efforts resulted in the development of MCD-KV-10, a novel thiazolo-thiophene derivative which showed good selectivity towards A<sub>2A</sub> and A<sub>3</sub> receptor subtypes. This compound was then screened for its *in vivo* mast cell stabilizing activity as it is well documented that degranulation of mast cells in diseased condition results in the release of number of constituents and *de novo* synthesized mediators which include histamine, leukotrienes and prostaglandins<sup>33</sup>. Therefore, mast cell stabilization is considered as a key factor in controlling the occurrence of asthma. Along with this, the compound was screened for its lipoxygenase (LOX) inhibition activity as LOX catalyses the conversion of arachidonic acid into leukotrienes which is also considered as an important inflammatory mediator leading to the progression and development of asthma<sup>34</sup>. Subsequently, it was evaluated for its *in vivo* efficacy against ovalbumin-induced chronic airway inflammation model in guinea pigs. The activity of the compound in the guinea pig model was quantified in terms of its effect on respiratory parameters, cytokine levels in broncho-alveolar lavage (BAL) fluid and serum, cellular counts in BAL fluid and blood, histamine level in lungs and histological evaluation of lung tissue.

## Materials and methods

### Chemicals

The reactions leading to the synthesis of MCD-KV-10 were carried on magnetic stirrer (Remi 2MLH, Mumbai, India) and heating stirrer (Radleys Discovery technology, RR98074, Essex, UK), as per the required experimental conditions. Buchi Rotavapor (R-210, Buchi India Private Ltd., Mumbai, India) was used for concentrating fractions under reduced pressure. Melting points were determined on a Veego VMP-DS apparatus (Veego, Mumbai, India). The FT-IR spectrum was recorded using IR Affinity 1 by Shimadzu, Kyoto, Japan. Mass spectra were obtained using LC-ESI-MS (Perkin Elmer mass spectrometer, Waltham, MA). The NMR spectra were recorded using Bruker biospin 500 MHz NMR spectrophotometer with chemical shift value represented in ppm relative to the internal standard tetramethyl silane. All reactions were monitored by TLC plates, pre-coated with silica gel 60 F<sub>254</sub> TLC plates (E. Merck, Darmstadt, Germany; 0.2 mm thickness) with aluminum sheet support and the compounds were detected by examination under UV lamp.

All the solvents and chemicals were obtained from Qualigens Fine Chemicals Ltd. (Mumbai, India) and were of analytical grade. Aluminium hydroxide gel was obtained from Merck Specialties Pvt. Ltd., Mumbai, India. *O*-Phthalaldehyde (OPT), acetylcholine (Ach), linoleic acid, lipoxygenase enzyme and histamine were purchased from M. P. Biomedicals, Solon, OH. Ovalbumin (OVA) was obtained from Himedia laboratories, Mumbai, India. Enzyme-linked immunosorbent assay (ELISA) kits for TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were purchased from Krishgen Biosystems, Mumbai, India for cytokine assay. Isoflurane (Raman and Weil Pvt. Ltd., Daman, India) was used for anesthetizing animals. Triple antigen was obtained from Biological E. Ltd., Hyderabad, India. Toluidine blue was obtained from Merck, Darmstadt, Germany. Quercetin was purchased from Sigma Aldrich, St. Louis, MO. Ketotifen fumarate was obtained as a gift sample from Torrent Pharmaceuticals, Ahmedabad, India. Dexamethasone was obtained from Cadila Pharmaceuticals, Ahmedabad, India. Deionized water used for

the experiment was prepared in-house using a water purification system (Millipore Elix, Darmstadt, Germany).

### Animals

Male *Dunkin–Hartley* guinea pigs weighing 300–400 g were obtained from the animal house of B. V. Patel PERD Centre, Ahmedabad, India. Animal housing and handling were performed in accordance with Good Laboratory Practice (GLP) mentioned in CPCSEA guidelines. The animal house is registered with the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Social Justice and Empowerment, Government of India, vide registration no. 1661/PO/a/12/CPCSEA, dated 21/11/2012. All experimental protocols were reviewed and accepted by the Institutional Animal Ethics Committee prior to initiation of the experiment. The animals were housed in stainless steel cages (three animals per cage) which were then placed in the experimental room where they were allowed to acclimatize for a week before experiment. A 10% air exhaust conditioning unit was maintained along with a relative humidity of 60  $\pm$  5% and a temperature of 25  $\pm$  3 °C in the animal house facility. A 10:14 h light:dark cycle was also regulated for the experimental animals. Amrut certified rodent diet (Maharashtra Chakan Oil Mill Ltd) and 0.01% vitamin C solution were provided *ad libitum* to the experimental animals.

### Synthesis and characterization of MCD-KV-10

MCD-KV-10 was synthesized by dissolving monochlorothiazole (A) (1 equivalent) in dimethylformamide, to which an enamine adduct (B), i.e. methyl 3-imino-2-(phenylcarbamothioyl) butanoate (1 equivalent) was added drop wise. The reaction was carried on a magnetic stirrer with constant stirring for 4 h. Progress of the reaction was monitored by TLC visualization under UV lamp, where the plate was developed in a mobile phase with a composition of ethyl acetate:hexane (3:7). The reaction mixture was added slowly along with continuous stirring into a beaker containing water. A yellow solid precipitate was obtained which was then filtered. The solid precipitate was given solvent retreatment to remove non-polar impurities and then recrystallized in dichloromethane to obtain MCD-KV-10. The detailed synthesis scheme for MCD-KV-10 is shown in Figure 1. The compound was then characterized by FT-IR, mass spectrum and NMR spectrum analysis.

### Adenosine receptor binding assay

The ligand affinity at A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> AR subtypes was determined by following the previously reported protocols<sup>35,36</sup>. Briefly, the membranes for radioligand binding were prepared from Chinese Hamster Ovary (CHO) cells stably transfected with human adenosine receptor subtypes. This sample was then centrifuged at 1000  $\times$  g for 10 min at 4 °C, to remove cell fragments and nuclei. This was followed by centrifugation at 100 000  $\times$  g for 1 h at 4 °C to obtain the crude membrane fraction. The resulting membrane pellets were then suspended in 50 mM Tris-HCl, pH 7.4 and assayed for radioligand binding. The radioligands for binding experiments were 1 nM [<sup>3</sup>H] CCPA for A<sub>1</sub> AR; 10 nM [<sup>3</sup>H] NECA for A<sub>2A</sub> receptor subtype; 1 nM [<sup>3</sup>H] HEMADO for A<sub>3</sub> receptor subtype. Non-specific binding of [<sup>3</sup>H] CCPA was determined in the presence of 1 mM of theophylline whereas R-phenylisopropyl adenosine was used for [<sup>3</sup>H] NECA and [<sup>3</sup>H] HEMADO. Inhibition of NECA stimulated adenylyl cyclase activity at A<sub>2B</sub> AR was determined as a measurement of affinity of the new compound. The K<sub>i</sub> values from the radioligand competition experiments were calculated using the program SCTFIT<sup>37</sup>. IC<sub>50</sub> values from adenylyl cyclase experiments were

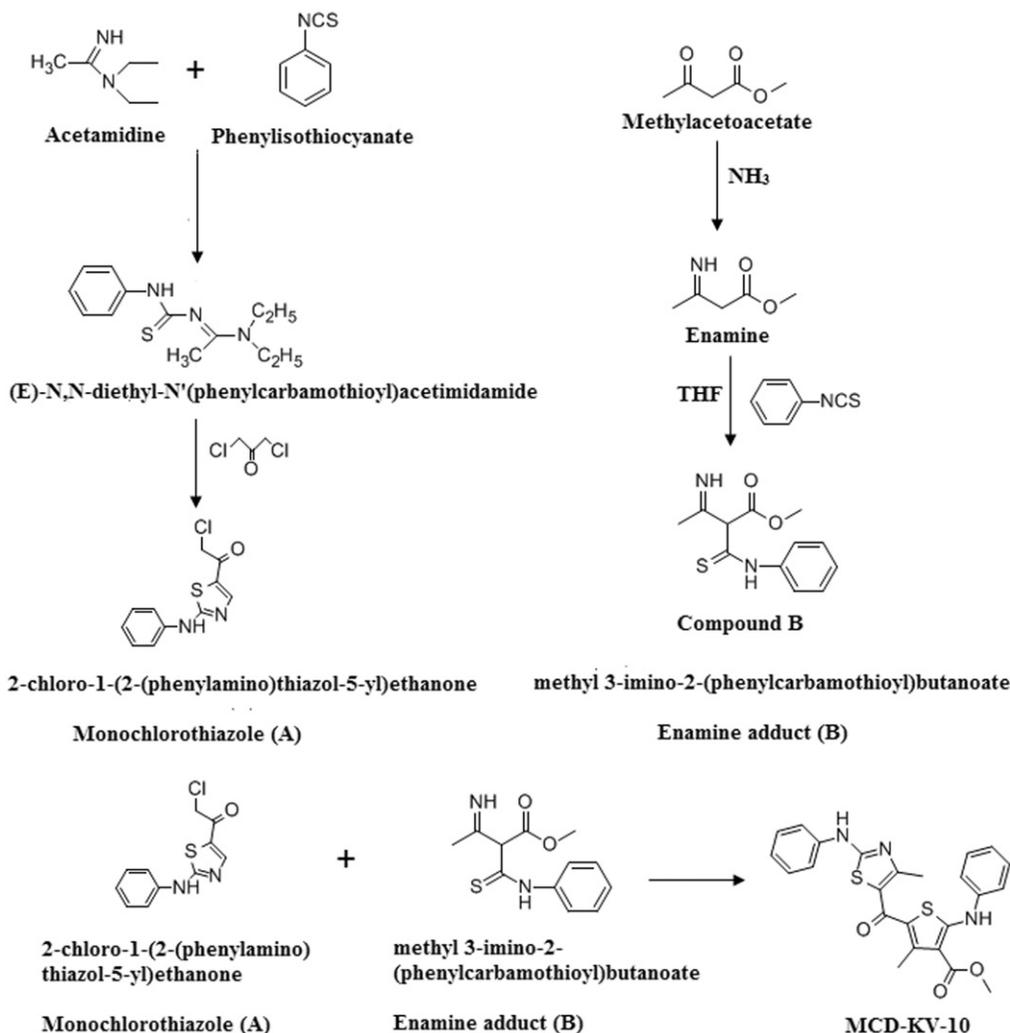


Figure 1. Synthesis scheme of MCD-KV-10. Synthesis of compound A: phenylisothiocyanate reacts with acetamide to form of an adduct which further reacts with dichloroacetone to form compound A. Synthesis of compound B: methylacetoacetate reacts with ammonia to form enamine which on subsequent reaction with phenylisothiocyanate forms compound B. Synthesis of MCD-KV-10: Compound A reacts with compound B in the presence of dimethylformamide at room temperature for 4 h to produce MCD-KV-10.

converted to  $K_i$  values using the equation derived by Cheng and Prusoff<sup>38</sup>.

### Lipoxygenase assay

The lipoxygenase assay was carried out as reported previously with some modifications<sup>39–41</sup>. MCD-KV-10 was screened at three different concentrations (25, 50 and 100  $\mu\text{g/mL}$ ). Absorbance readings were collected at 254 nm. Quercetin (Concentration: 15  $\mu\text{g/mL}$ ) was used as a positive control. The percentage inhibition of lipoxygenase was calculated using formula:

$$\text{Percentage inhibition} = \left[ \frac{(\text{absorbance of blank} - \text{absorbance of test})}{\text{absorbance of test}} \right] \times 100.$$

### Mast cell stabilization assay

The mast cell stabilization activity was carried out as reported previously<sup>42</sup>. Briefly, guinea pigs were sensitized by injecting subcutaneously 0.5 mL sheep serum along with 0.5 mL of triple antigen. Animals were divided into three groups (six animals per group). Group I served as a control (non-sensitized) and received vehicle (0.2% agar solution), Group II (sensitized disease control group received 0.2% agar solution), Group III (Positive control

received ketotifen fumarate), Dose: 1 mg/kg bodyweight, orally), Group IV (Treatment with MCD-KV-10, Dose: 20 mg/kg bodyweight, orally). All the drugs were administered orally as a suspension in 0.2% of agar. Treatment was continued for 14 days. On the 14th day, 2 h after the assigned treatment, animals were sacrificed; intestinal mesenteries were taken out and kept in Ringer Locke solution for 30 min. These mesenteric fans were challenged with 5% sheep serum for 10 min and then fixed, dried, stained with toluidine blue and examined microscopically for the number of intact and degranulated mast cells in at least 20 randomly selected high power fields. The percentage inhibition of the mast cell degranulation was calculated by using formula:

$$\% \text{Inhibition of mast cell degranulation} = \left[ \frac{(100 - \text{number of degranulated mast cells})}{\text{total number of mast cells}} \right] \times 100$$

### Evaluation of anti-asthmatic activity in ova-albumin induced airway inflammation in guinea pigs

#### Sensitization and treatment of animals

Guinea pigs used in this study were divided into four different groups of six animals each: group I, non-sensitized controls

received 0.2% agar solution (control group); group II (disease control group), OVA sensitized and then received 0.2% agar solution orally (vehicle for dexamethasone [DXM] and MCD-KV-10 treatments); group III, OVA sensitized and then received DXM (Dose: 2.5 mg/kg body weight orally) as reference standard; group IV, OVA sensitized and then received MCD-KV-10 (Dose: 20 mg/kg body weight orally). All animals (except group I) were sensitized following a previously reported method<sup>43</sup>, using a subcutaneous injection of 100 mg of ovalbumin (which had been adsorbed onto 100 mg of aluminum hydroxide in saline) on day 0 as the first sensitization. Second sensitization was then carried out using the same dose of antigen 2 weeks later (i.e. on day 14). The daily doses of drug or vehicle were initiated on day 18 and continued until day 21 by oral route.

### Ovalbumin exposure

The animals were challenged with 0.5% of aerosolized OVA for 10 min, 2.5 h after receiving their drug or vehicle treatment, from day 18 to day 21. For the challenge, conscious animals were placed into a plastic circular chamber (diameter  $\frac{1}{4}$  70 cm, height  $\frac{1}{4}$  40 cm) connected to a nebulizer (CX4-Omron Healthcare, Kyoto, Japan). Animals in the non-sensitized group (group I) were exposed to aerosolized saline following the same protocol.

### Lung function and bronchoconstriction test

On day 21, tidal volume and respiration rate of animals were measured after 2 h of 10 min of OVA exposure using a data acquisition system (MP-35, Biopac Systems, Santa Barbara, CA). The means to measure airway resistance directly was unavailable hence; the changes in respiratory rate and tidal volume were used as an indirect surrogate. All animals were subjected to a bronchoconstriction test immediately, after measurement of lung function parameters, where all OVA-sensitized animals were exposed to 0.25% Acetylcholine (Ach) solution for 30 s using a nebulizer (CX4-Omron Healthcare). Guinea pigs in the non-sensitized control group were exposed to normal saline in place of Ach. Tidal volume and respiration rate of each animal were recorded before and after Ach exposure.

### Cellular count and serum preparation

On day 22, blood samples (3 mL) were collected from all the animals under isoflurane anesthesia. Each sample was then divided into two portions. The first aliquot (2.5 mL) was placed in a non-heparinized tube for serum; the separated serum was stored at  $-80^{\circ}\text{C}$  for later quantitative determination of cytokines. The second portion (0.5 mL) was placed in a heparinized tube and used for total and differential leukocyte counts. Detailed procedures for total and differential leukocyte counts were the same as those described for the BAL fluid (see below).

### Bronchoalveolar lavage

At the end of experiment (i.e. day 22), all the animals were anaesthetized with isoflurane for the collection of BAL fluid. A polypropylene cannula was then inserted into the trachea, and normal saline solution (10 mL), maintained at  $37^{\circ}\text{C}$  was introduced into lungs via a 10-mL syringe and then collected 5 min later. The recovered lavage fluid (5 mL) was centrifuged at  $500 \times g$  for 10 min at  $4^{\circ}\text{C}$ ; the resultant supernatant was collected and stored at  $-80^{\circ}\text{C}$  until used in cytokine determinations. The cells in the pellet were washed in 0.5 mL of normal saline and total cell counts were then performed in an automated hematology analyzer (Abaxis Inc., Union City, CA).

### Cytokines in serum and BAL fluid

The levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in collected serum and BAL samples were measured using ELISA kits. The standard and samples were processed following the manufacturer's protocol and all plates were analyzed on an automated plate reader (BIOTEK ELx800-MS ELISA Plate Reader, India). Linearity for the calibration curve was found in the range of 62.5–2000 pg/mL as mentioned in the manufacturer's protocol.

### Histamine assay in lavaged lung tissue

Lung tissue lobe from each animal was dissected out separately immediately following BAL fluid collection. Lung tissue ( $500 \pm 20$  mg) was placed in 2.5 mL of normal saline for the preparation of homogenate. The presence of histamine was determined fluorometrically (SL-174, Elico, India) by the modified method of Shore et al.<sup>44</sup>.

### Histological evaluation of lung tissue

Histological studies were done on excised lung tissue of guinea pigs from all animal groups. Five micrometer sectioning of the lung was done using a rotary microtome (Model 0126, Yorco, India) followed by staining with haematoxylin and eosin. Tissue sections were observed for all possible changes in the anatomy under Olympus IX51 optical microscope (Olympus Optical Co. Ltd., Tokyo, Japan). The photographs were taken with an Olympus TL4 camera. The observations were compared to detect significant histological changes, if any.

### Statistical analysis

Statistical analysis was performed using One-way ANOVA followed by Bonferroni's multiple comparison test where probability values,  $p \leq 0.05$  were considered to be significant. The activity of normal control, positive control and test compounds were compared with disease control group.

## Results

### Characterization of the designed molecules

The characterization of the synthesized compound was done using; FT-IR, mass spectra analysis and proton NMR data (data represented in supplementary material) and yields were calculated. The characterization data of MCD-KV-10 is discussed below.

#### MCD-KV-10

Chemical formula: Methyl-4-methyl-5-(4-methyl-2-(phenyl amino)thiazole-5-carbonyl)-2-(phenyl amino)thiophene-3-carboxylate.

Yield: 58.32%; molecular formula:  $\text{C}_{24}\text{H}_{21}\text{N}_3\text{O}_3\text{S}_2$ ; yellow color solid; molecular weight: 463 g/mol; LC-ESI-MS ( $m/z$ ): 464 ( $M+1$ )<sup>+</sup>; melting point:  $155\text{--}156^{\circ}\text{C}$ ;  $R_f$ : 0.35 (hexane:ethyl acetate – 7:3); IR (KBr,  $\text{cm}^{-1}$ ): 3213.41 (NH stretch), 1587.42 (NH bend), 1660.50 (O=C=O), 1658.78 (C=O), 1537.27 (C=C), 3176.76 (aromatic CH stretch), 1238.30 (C–O stretch); <sup>1</sup>H-NMR: (500 MHz, DMSO- $d_6$ ): 10.7 (s, 1H), 10.06 (s, 1H), 7.67–7.61 (m, 3H), 7.41–7.31 (m, 4H), 7.19–7.11 (m, 3H), 4.01 (s, 3H), 2.48 (s, 3H), 2.42 (s, 3H).

### Adenosine receptor binding assay

MCD-KV-10 showed the highest affinity for  $A_3$ -ARs ( $K_i = 797$  nM) followed by  $A_{2A}$  binding with a  $K_i$  of 4820 nM. For the  $A_1$  subtype,  $K_i$  value of 9680 nM was determined, whereas limited solubility prevented an exact determination of  $A_{2B}$  affinity.

( $K_i > 10\,000$  nM). The detailed radioligand binding data is shown in Table 1.

### Lipoxygenase assay

It has been reported that compounds inhibiting lipoxygenases can be employed in the treatment of allergy/asthma<sup>39</sup>. Based on these evidences, MCD-KV-10 was also evaluated for its ability to inhibit lipoxygenase enzyme at three different concentrations 25, 50 and 100  $\mu\text{g}/\text{mL}$ . The compound showed inhibitory activity at 25, 50 and 100  $\mu\text{g}/\text{mL}$  with percentage inhibition value of 38.1, 46.8 and 57.1%, respectively. The percentage inhibition for the positive control quercetin at 15  $\mu\text{g}/\text{mL}$  was 61.9%.

### Mast cell stabilization assay

During degranulation, mast cells release a number of mediators which include histamine, leukotrienes and prostaglandins. Therefore, mast cell stabilization is a key factor in controlling the occurrence of asthma<sup>33</sup>. Mast cell degranulation data as shown in Figure 2 implicates that MCD-KV-10, at dose 20 mg/kg showed significant protection ( $p \leq 0.05$ ) to mast cells degranulation ( $66.0 \pm 2.8\%$ ) when compared to the untreated disease group animals. Figure 3 shows the difference between the intact and degranulated mast cell in different animal groups.

### Evaluation of anti-asthmatic activity in ova-albumin induced airway inflammation in guinea pigs

#### Effect of MCD-KV-10 on body weight

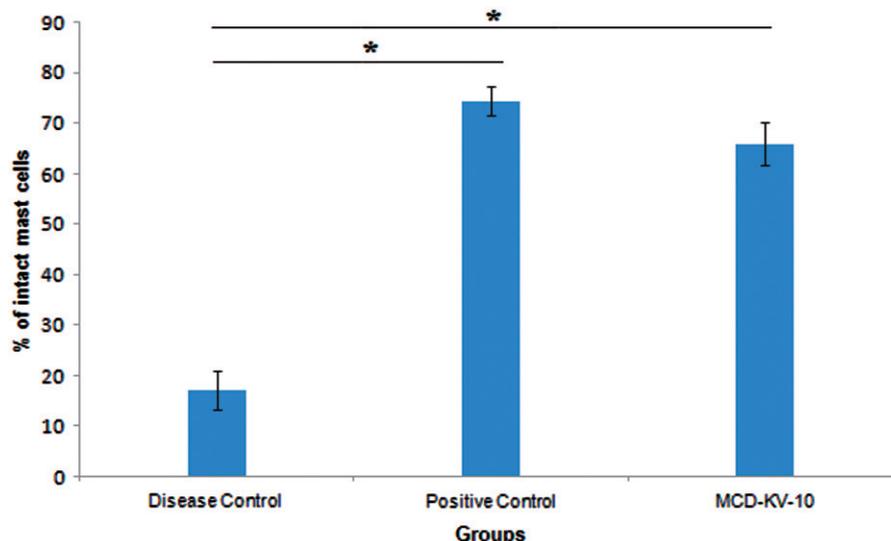
No animals in the disease control group (group II) and drug regimen groups (groups III and IV) exhibited any significant differences in body weight compared with those of the

Table 1.  $K_i$  values from radioligand binding experiment for MCD-KV-10 at  $A_1$ ,  $A_{2A}$  and  $A_3$  receptor subtypes.

Compound	Adenosine receptor subtypes		
	$A_1$	$A_{2A}$	$A_3$
MCD-KV-10	9680 (6190–15 100)	4820 (3770–6180)	797 (564–1130)

Values are  $K_i$  values and are given in nM with 95% confidence intervals in parentheses.

Figure 2. Mast cell degranulation in intestinal mesenteric tissues of guinea pigs. Data expressed as mean  $\pm$  SD ( $n = 6$ ).



non-sensitized controls during the experimental period (data not shown). Furthermore, there were no apparent effects on appetite/water consumption or outward appearance (i.e. fur coat, eyes) of these animals in each treatment group.

#### Effect of MCD-KV-10 on lung function and bronchoconstriction test

It was observed that MCD-KV-10 prevents the changes in respiratory parameters due to acetylcholine inhalation observed in the disease control group. MCD-KV-10 inhibited the increase in respiratory rate significantly after acetylcholine challenge as shown in Figure 4. A similar response was observed in case of tidal volume measurement when the compound prevented the significant decrease in tidal volume as seen in the disease control group (Figure 5).

#### Effect of MCD-KV-10 on cellular count in blood and BAL fluid

Infiltration of proinflammatory cells into the airways is an important characteristic of asthma<sup>45</sup>. The infiltration of proinflammatory cells into the airways was evaluated by collection of BAL fluid after 24 h of last provocation. Table 2 shows the total WBC count in BAL fluid of different animal groups. The results of differential count of BAL fluid samples showed a significant difference ( $p \leq 0.05$ ) in lymphocyte and neutrophil counts among the animals of different groups as shown in Table 3. Total WBC count in blood was done on day 17 as a baseline and on day 22 as shown in Table 2 which also showed significantly high WBC count ( $p \leq 0.05$ ) in disease control group as compared to other groups.

#### Effect of MCD-KV-10 on cytokine production in serum and BAL fluid

The airway inflammation underlying asthma is regulated by a network of mutually interacting cytokines. However, the exact functional role of each individual cytokine in the pathogenesis of the disease remains to be completely recognized<sup>46</sup>. Levels of IL-1 $\beta$  in BAL fluids of patients with asthma were found to be increased compared with those of non-asthmatic individual. TNF- $\alpha$  may have an important amplifying effect on asthmatic inflammation and it is proof that its expression is increased in asthmatic airways. Role of IL-6 in asthma pathophysiology is still controversial. It has been accepted that IL-6 is released from alveolar macrophages from asthmatic patients after allergen challenge and increased basal release, compared with

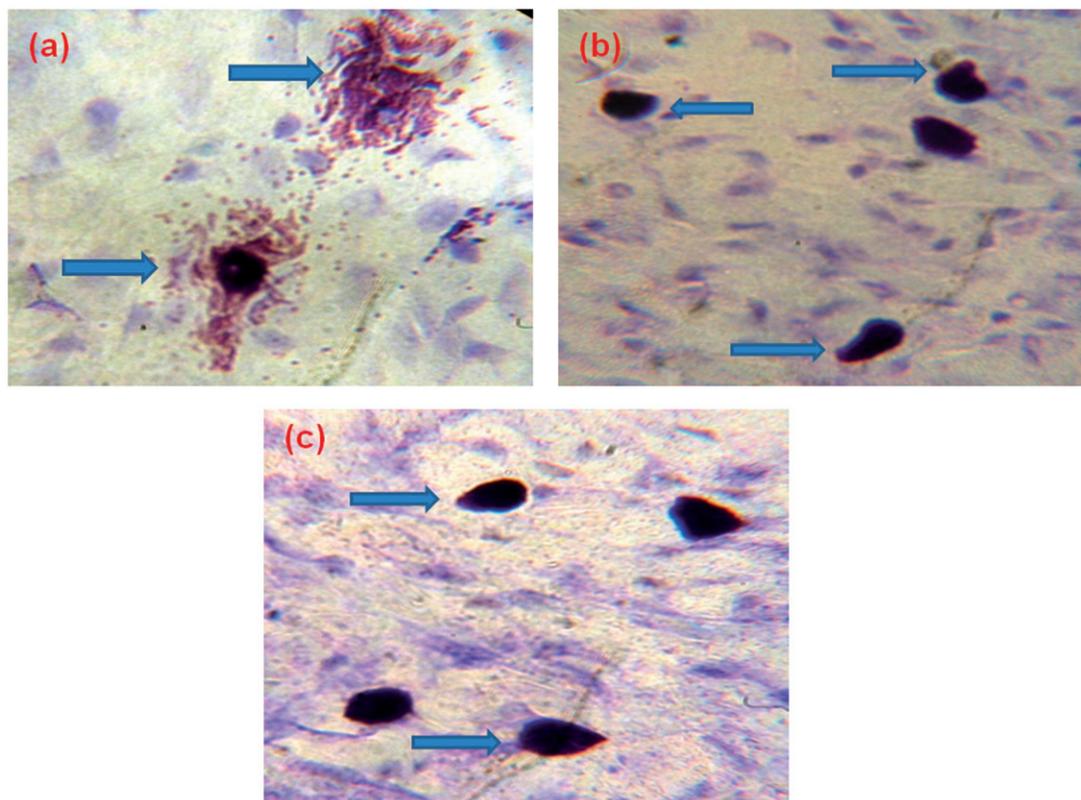
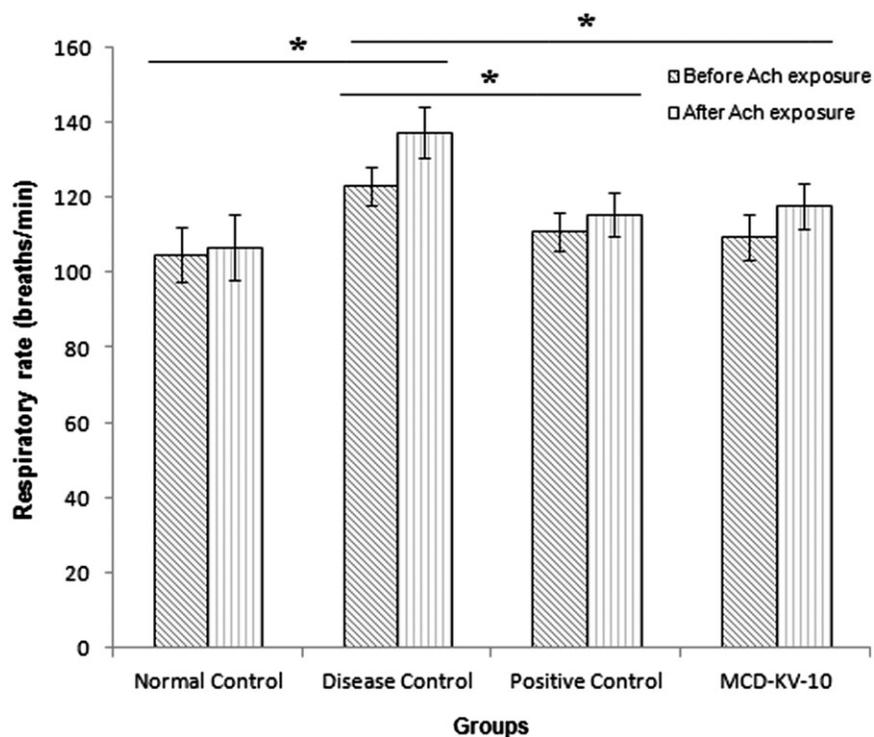


Figure 3. Intact and degranulated mast cells in the intestinal mesenteric tissues of (a) disease control, (b) positive control and (c) test group (MCD-KV-10) animals.

Figure 4. Respiratory rate in guinea pigs of all groups before and after acetylcholine (ACh) challenge. Data expressed as mean  $\pm$  SD ( $n=6$ ). \* $p \leq 0.05$  as compared to disease control group.



non-asthmatic subjects<sup>47,48</sup>. The levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in serum (Figure 6) and BAL fluid (Figure 7) of guinea pigs of disease control group were significantly ( $p \leq 0.05$ ) higher than those in the non-sensitized controls, positive control and test group (animals treated with MCD-KV-10). DXM and MCD-KV-10 treatment significantly reduced TNF- $\alpha$ , IL-1 $\beta$  and IL-6 levels as compared with the disease controls.

#### Effect of MCD-KV-10 on histamine levels

Histamine levels in lung homogenates of the previously lavaged lungs were assayed (Figure 8). The levels of histamine in disease control animals were significantly ( $p \leq 0.05$ ) higher than those in the non-sensitized controls. Both positive control and treatment with MCD-KV-10 showed a significant ( $p \leq 0.05$ ) decrease

Figure 5. Tidal volume in guinea pigs of all groups before and after acetylcholine (ACh) challenge. Data expressed as mean  $\pm$  SD ( $n=6$ ). \* $p \leq 0.05$  as compared to disease control group.

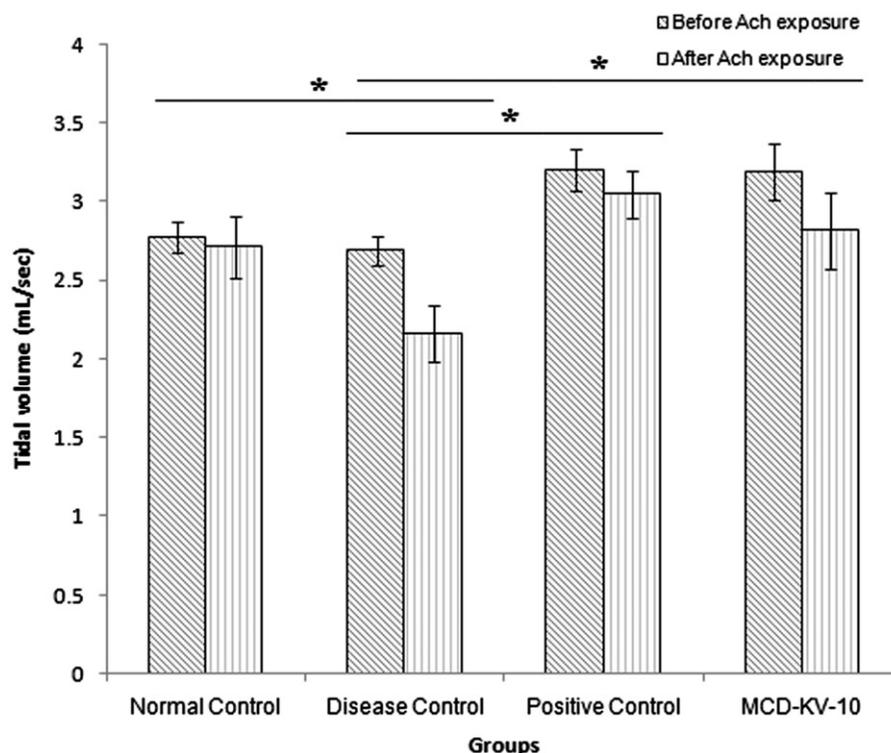


Table 2. Total WBC count in blood and BAL fluid of guinea pigs.

Groups	WBC in blood ( $\times 10^{-3}/\mu\text{L}$ )		WBC in BAL fluid ( $\times 10^{-3}/\mu\text{L}$ )
	Day 17	Day 22	Day 22
Normal control	8.03 $\pm$ 0.35	8.525 $\pm$ 0.48	7.48 $\pm$ 1.17
Disease control*	9.45 $\pm$ 0.31	15.475 $\pm$ 2.01	19.08 $\pm$ 1.82
Positive control	9.40 $\pm$ 0.84	7.95 $\pm$ 1.98	7.25 $\pm$ 0.97
MCD-KV-10	11.73 $\pm$ 0.80	8.825 $\pm$ 1.54	8.45 $\pm$ 1.02

Data expressed as mean  $\pm$  SD, ( $n=6$ ).

\* $p \leq 0.05$  as compared to disease control group.

Table 3. Differential count in BAL fluid of guinea pigs.

Groups	Lymphocytes	Neutrophils	Mixed
Normal control	61.08 $\pm$ 1.86	36.58 $\pm$ 2.65	2.34 $\pm$ 0.39
Disease control*	34.75 $\pm$ 4.68	53.68 $\pm$ 4.89	11.57 $\pm$ 1.51
Positive control	59.76 $\pm$ 2.38	32.28 $\pm$ 2.17	7.96 $\pm$ 1.29
Test group (MCD-KV-10)	54.55 $\pm$ 3.57	38.23 $\pm$ 2.57	7.22 $\pm$ 1.11

Data expressed as mean  $\pm$  SD, ( $n=6$ ).

\* $p \leq 0.05$  as compared to disease control group.

in the degree of histamine release that was significantly increased in disease control animals compared to the normal control.

### Histological evaluation of lung tissue

Histological evaluation of lungs of guinea pigs as shown in Figure 9 showed no significant difference amongst different animal groups.

### Discussion

Allergic mediators are very well known to exert individual effects which combine to generate asthma symptoms and may also interact to produce the inflammatory characteristics of the disease. These interactions subsequently lead to bronchial

hyperactivity and increased release of mediators from inflammatory cells, as well as for the generation of bronchospasm<sup>49–52</sup>. The receptor-based studies pertaining to the development and progression of asthma show involvement of many targets of which adenosine receptor subtypes have a significant contribution. Thus realizing the significant involvement of AR in asthma, we tried to develop a potential lead which may be used for management of asthma by targeting the adenosine receptor subtypes. We synthesized the novel thiazolo-thiophene derivative, MCD-KV-10. Prior to its *in vivo* pharmacological evaluation, we conducted *in vitro* studies to determine the ligand binding affinity at AR subtypes using membranes from Chinese Hamster Ovary (CHO) cells stably transfected with the human adenosine receptor subtypes. The results showed that this compound has highest affinity and selectivity towards  $A_{2A}$  and  $A_3$  AR receptor subtypes. Along with this, the compound was evaluated for its *in vitro* lipoxygenase inhibition activity because lipoxygenase-derived metabolic products interact with other mediators for the production of asthmatic symptoms<sup>39</sup>. The compound showed good lipoxygenase inhibition activity with an inhibition of 57.1% at a concentration of 100  $\mu\text{g}/\text{mL}$ .

Based on these promising results, the compound was evaluated for its *in vivo* pharmacological activity. We initially started with evaluation of its potential for inhibition of mast cell degranulation in intestinal mesenteries of guinea pigs. MCD-KV-10 (Dose: 20 mg/kg) showed significant protection to mast cells degranulation (66.0  $\pm$  2.8%), which was considered as an important finding for this compound to have potential anti-asthmatic activity. Finally, the compound was screened for its anti-asthmatic activity using ovalbumin-induced airway inflammation in a guinea pig model of asthma. The animals in the test group were dosed with MCD-KV-10 (20 mg/kg bodyweight). Dexamethasone (5 mg/kg bodyweight) was used at positive control in this experiment. Initially, we determined the effect of treatment on hyper-responsiveness by evaluating the lung function in acetylcholine induced bronchoconstriction test. This was done as it is well documented that hyper-responsiveness is another important characteristic of asthma and widely used as efficacious tool for

Figure 6. Effect of treatments on serum cytokine levels of guinea pigs. Data expressed as mean  $\pm$  SD, ( $n=6$ ). \* $p \leq 0.05$  as compared to disease control group.

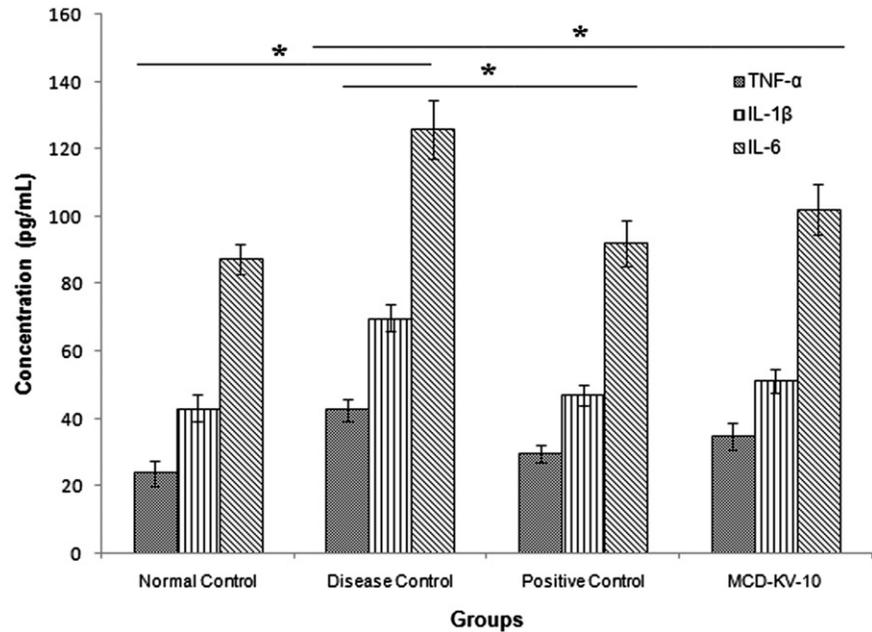
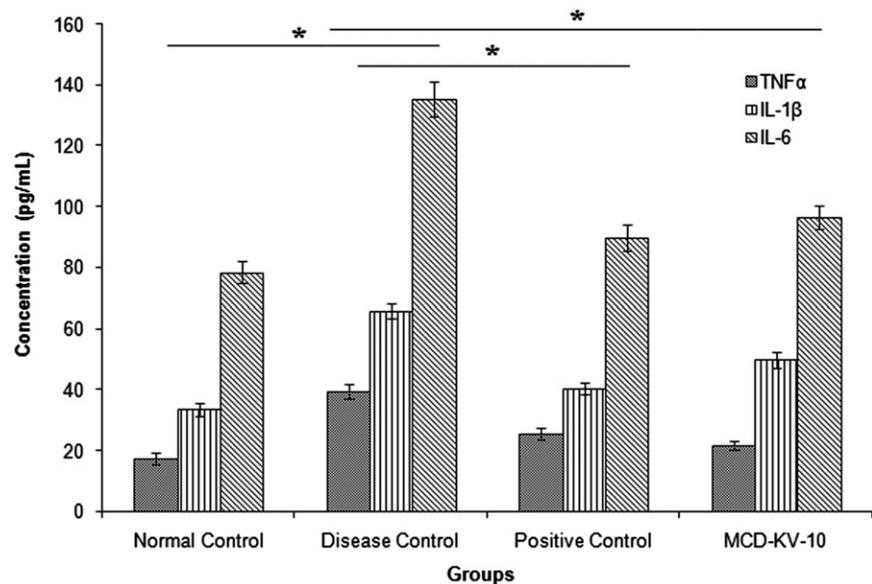


Figure 7. Effect of treatments on BAL fluid cytokine levels of guinea pigs. Data expressed as mean  $\pm$  SD, ( $n=6$ ). \* $p \leq 0.05$  as compared to disease control group.



pharmacological screening of various anti-asthmatic agents in animal model of asthma<sup>53</sup>. The results showed that MCD-KV-10 prevented the increase in respiratory rate and decrease in tidal volume, which were significantly different as compared to disease control animals.

An upsurge of pro-inflammatory cells is associated with asthma. These generally infiltrate into the airways and are present in the circulation which can be monitored by determining WBC count and differential counts in the BAL fluid and blood samples<sup>54</sup>. We found that the animals treated with MCD-KV-10 showed a significantly lower WBC count in both BAL fluid and blood samples as compared to the disease control group at the end of the study period indicating its potential to control the rise and infiltration of pro-inflammatory cells in the airways.

It is well-documented that allergen-specific Th2 responses with the subsequent release of cytokines are responsible for the cascade of events resulting in asthma<sup>55</sup>. Thus, it may be presumed that a potential anti-asthmatic compound should be able to reduce cytokine levels. In our study, we determined the levels of three primary cytokines, namely, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in BAL

fluid and serum samples as previous reports suggest their primary involvement in the pathophysiology of asthma. The results obtained were quite promising as the animals treated with MCD-KV-10 showed significantly lower cytokines levels as compared to the disease control animals in both BAL fluid and serum samples. However, no significant differences in cytokine levels were observed among the test and positive control groups.

Along with the cytokines, histamine is also considered as an important marker in asthma. Previous reports emphasize the importance of histamine in the development of the asthmatic response, and show that newer antihistamines might be helpful to reduce asthma symptoms<sup>48</sup>. Based on these findings, we evaluated the anti-histaminic potential of MCD-KV-10 in the same guinea pig model as discussed above. The results inferred that animals treated with MCD-KV-10 showed significantly lower histamine concentration in lung tissues as compared to the disease control animals, thus confirming its anti-histaminic potential. However, the histamine levels in positive control animals were comparable to that of test group. This study further emphasized the effect of MCD-KV-10 to inhibit histamine release. The result

Figure 8. Effect of treatments on lung histamine levels of guinea pigs. Data expressed as mean  $\pm$  SD, ( $n = 6$ ). \* $p \leq 0.05$  as compared to disease control group.

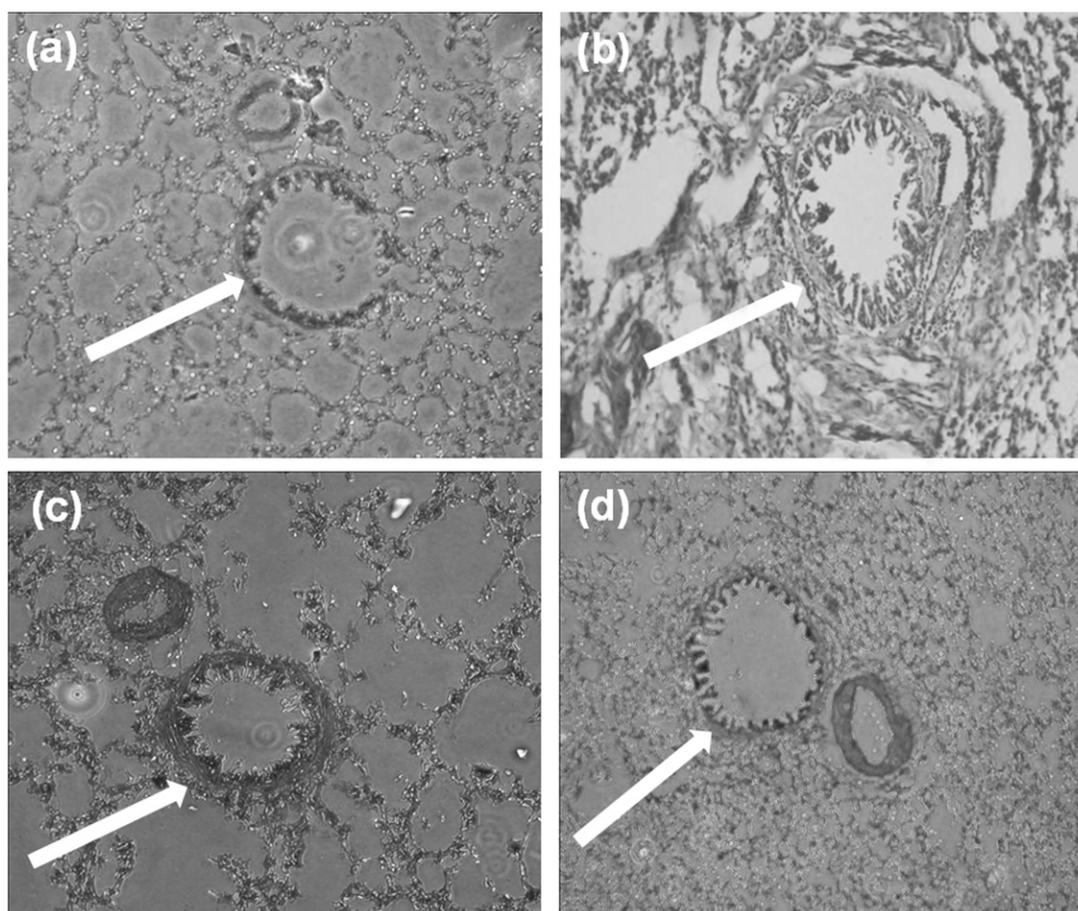
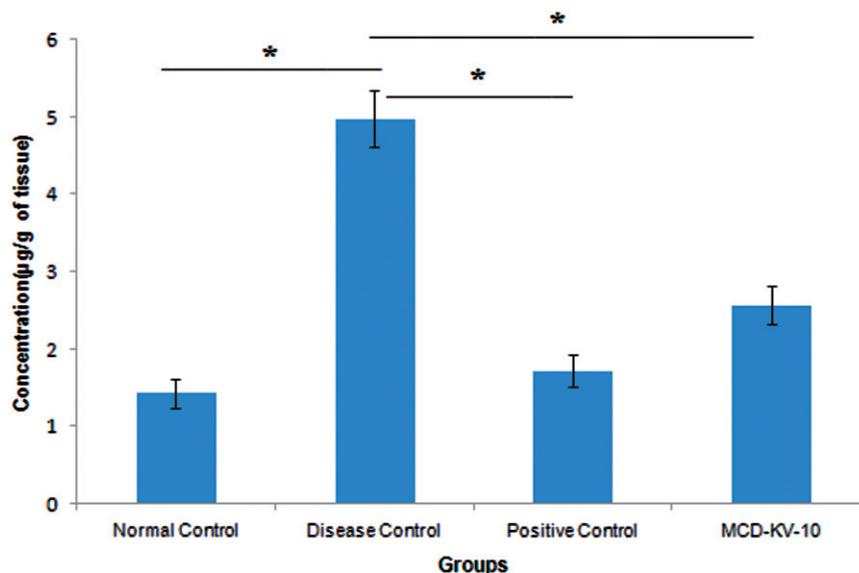


Figure 9. Histomicrographs of lung tissue of (a) normal control, (b) disease control, (c) positive control and (d) test group (MCD-KV-10) animals.

of histological evaluation of the lungs of guinea pigs showed no significant difference among different animal groups.

### Conclusion

The findings of this study suggest regarding the probable implication of MCD-KV-10 in management of asthma, by primarily modulating  $A_{2A}$  and  $A_3$ -AR receptor subtypes. In this study, we highlighted lipoxygenase inhibition and mast cell

stabilization potential of MCD-KV-10 where it showed good activity. MCD-KV-10 also showed good anti-asthmatic potential in guinea pig model of asthma, which was confirmed from its efficacy against T-cell-derived cytokine production, subsequent cytokine-mediated cellular infiltration, its bronchodilatory potential and capacity to block mediator release into the local tissues like lung. This study gave an implication for exploration of thiazolo-thiophene class of compounds with other substitutions for management of asthma. However, further mechanistic studies

in future will give us a better insight about the role of this class of compounds on other targets and inflammatory mediators underlying asthma.

### Acknowledgements

The authors wish to acknowledge NIPER – Ahmedabad for providing all the facilities to carry out this work and Dr. Sonja Kachler and Dr. Karl-Norbert Klotz from Institut für Pharmakologie und Toxikologie, Julius-Maximilians-Universität, Germany for their assistance in *in vitro* studies. The authors are also grateful to Torrent Pharmaceuticals (Ahmedabad, India) for providing ketotifen fumarate as a gift sample.

### Declaration of interest

The authors declare that there are no conflicts of interest.

### References

- Fredholm BB, IJzerman AP, Jacobson KA, et al. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* 2001;53:527–52.
- Fredholm BB, Abbracchio MP, Burnstock G, et al. Nomenclature and classification of purinoceptors. *Pharmacol Rev* 1994;46:143–6.
- Martin RJ, Kraft M, Chu HW, et al. A link between chronic asthma and chronic infection. *J Allergy Clin Immunol* 2001;107:595–601.
- Meade CJ, Worrall L, Hayes D, Protin U. Induction of interleukin 8 release from the HMC-1 mast cell line: synergy between stem cell factor and activators of the adenosine A<sub>2</sub>(b) receptor. *Biochem Pharmacol* 2002;64:317–25.
- Cronstein BN, Daguma L, Nichols D, et al. The adenosine/neutrophil paradox resolved: human neutrophils possess both A<sub>1</sub> and A<sub>2</sub> receptors that promote chemotaxis and inhibit O<sub>2</sub> generation, respectively. *J Clin Invest* 1990;85:1150–7.
- Cronstein BN, Levin RI, Philips M, et al. Neutrophil adherence to endothelium is enhanced *via* adenosine A<sub>1</sub> receptors and inhibited *via* adenosine A<sub>2</sub> receptors. *J Immunol* 1992;148:2201–6.
- Salmon JE, Cronstein BN. Fc gamma receptor-mediated functions in neutrophils are modulated by adenosine receptor occupancy. A<sub>1</sub> receptors are stimulatory and A<sub>2</sub> receptors are inhibitory. *J Immunol* 1990;145:2235–40.
- Marone G, Petracca R, Vigorita S, et al. Adenosine receptors on human leukocytes IV. Characterization of an A<sub>1</sub>/R<sub>1</sub> receptor. *Int J Clin Lab Res* 1992;22:235–42.
- Salmon JE, Brogle N, Brownlie C, et al. Human mononuclear phagocytes express adenosine A<sub>1</sub> receptors. A novel mechanism for differential regulation of Fc gamma receptor function. *J Immunol* 1993;151:2775–85.
- Ahmed AH, Jacobson KA, Kim J, Heppel LA. Presence of both A<sub>1</sub> and A<sub>2A</sub> adenosine receptors in human cells and their interactions. *Biochem Biophys Res Commun* 1995;208:871–8.
- Peachell PT, Columbo M, Kagey-Sobotka A, et al. Adenosine potentiates mediator release from human lung mast cells. *Am Rev Respir Dis* 1988;138:1143–51.
- Forsythe P, McGarvey LPA, Heaney LG, et al. Adenosine induces histamine release from human bronchoalveolar lavage mast cells. *Clin Sci* 1999;96:349–55.
- Murakami S, Hashikawa T, Saho T, et al. Adenosine regulates the IL-1 $\beta$  induced cellular functions of human gingival fibroblasts. *Int Immunol* 2001;13:1533–40.
- Panther E, Idzko M, Herouy Y, et al. Expression and function of adenosine receptors in human dendritic cells. *FASEB J* 2001;15:1963–70.
- Wilson CN, Batra VK. Lipopolysaccharide binds to and activates A<sub>1</sub> adenosine receptors on human pulmonary artery endothelial cells. *J Endotoxin Res* 2002;8:263–71.
- McNamara N, Gallup M, Khong A, et al. Adenosine up-regulation of the mucin gene, MUC2, in asthma. *FASEB J* 2004;18:1770–2.
- Ethier MF, Madison JM. Adenosine A<sub>1</sub> receptors mediate mobilization of calcium in human bronchial smooth muscle cells. *Am J Respir Cell Mol Biol* 2006;35:496–502.
- Clark AN, Youkey R, Liu X, et al. A<sub>1</sub> adenosine receptor activation promotes angiogenesis and release of VEGF from monocytes. *Circ Res* 2007;101:1130–8.
- Brown RA, Clarke GW, Ledbetter CL, et al. Elevated expression of adenosine A<sub>1</sub> receptor in bronchial biopsies from asthmatic subjects. *Eur Respir J* 2008;31:1–9.
- Bjorck T, Gustafsson LE, Dahlen SE. Isolated bronchi from asthmatics are hyper responsive to adenosine, which apparently acts indirectly by liberation of leukotrienes and histamine. *Am Rev Respir Dis* 1992;145:1087–91.
- Hughes PJ, Holgate ST, Church MK. Adenosine inhibits and potentiates IgE-dependent histamine release from human lung mast cells by an A<sub>2</sub>-purinoceptor mediated mechanism. *Biochem Pharmacol* 1984;33:3847–52.
- Peachell PT, Lichtenstein LM, Schleimer RP. Differential regulation of human basophil and lung mast cell function by adenosine. *J Pharmacol Exp Ther* 1991;256:717–26.
- Suzuki H, Takei M, Nakahata T, Fukamachi H. Inhibitory effect of adenosine on degranulation of human cultured mast cells upon cross-linking of Fc epsilon RI. *Biochem Biophys Res Commun* 1998;242:697–702.
- Felsch A, Stocker K, Borchard U. Phorbol ester-stimulated adherence of neutrophils to endothelial cells is reduced by adenosine A<sub>2</sub> receptor agonists. *J Immunol* 1995;155:333–8.
- Wollner A, Wollner S, Smith JB. Acting *via* A<sub>2</sub> receptors, adenosine inhibits the upregulation of Mac-1 (Cd11b/CD18) expression on FMLP-stimulated neutrophils. *Am J Respir Cell Mol Biol* 1993;9:179–85.
- Fredholm BB, Zhang Y, Van der Ploeg I. Adenosine A<sub>2A</sub> receptors mediate the inhibitory effect of adenosine on formyl-Met-Leu-Phe-stimulated respiratory burst in neutrophil leucocytes. *Naunyn Schmiedeberg's Arch Pharmacol* 1996;354:262–7.
- Hannon JP, Bray-French KM, Phillips RM, Fozard JR. Further pharmacological characterization of the adenosine receptor subtype mediating inhibition of oxidative burst in human isolated neutrophils. *Drug Dev Res* 1998;43:214–24.
- Bouma MG, Jeunhomme TM, Boyle DL, et al. Adenosine inhibits neutrophil degranulation in activated human whole blood: involvement of adenosine A<sub>2</sub> and A<sub>3</sub> receptors. *J Immunol* 1997;158:5400–8.
- Huang S, Apasov S, Koshiba M, Sitkovsky M. Role of A<sub>2A</sub> extracellular adenosine receptor-mediated signaling in adenosine mediated inhibition of T-cell activation and expansion. *Blood* 1997;90:1600–6.
- Fishman P, Bar-Yehuda S. Pharmacology and therapeutic applications of A<sub>3</sub> receptor subtypes. *Curr Topics Med Chem* 2003;3:463–9.
- Nadeem A, Mustafa SJ. Adenosine receptor antagonists and asthma. *Drug Discov Today Ther Strat* 2006;3:269–75.
- Scheiff AB, Yerande SG, Tayeb AE, et al. 2-Amino-5-benzoyl-4-phenylthiazoles: development of potent and selective adenosine A<sub>1</sub> receptor antagonists. *Bioorg Med Chem* 2010;18:2195–203.
- Slater JW, Zechin AD, Haxby DG. Second generation antihistamines: a comparative review. *Drugs* 1999;57:31–47.
- Gilman A, Goodman L, Rall T, Murad F. Goodman and Gilman's – the pharmacological basis of therapeutics. USA: Macmillan; 2010:659–60.
- Klotz KN, Hessling J, Hegler J, et al. Comparative pharmacology of human adenosine receptor subtypes – characterization of stably transfected receptors in CHO cells. *Naunyn-Schmiedeberg's Arch Pharmacol* 1998;357:1–9.
- Klotz KN, Falgner N, Kachler S, et al. [<sup>3</sup>H]HEMADO – a novel tritiated agonist selective for the human adenosine A<sub>3</sub> receptor. *Eur J Pharmacol* 2007;556:14–18.
- De Lean A, Hancock AA, Lefkowitz RJ. Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. *Mol Pharmacol* 1982;21:5–16.
- Cheng Y, Prusoff WH. Relationship between the inhibition constant (K<sub>i</sub>) and the concentration of inhibitor which causes 50 per cent inhibition (I<sub>50</sub>) of an enzymatic reaction. *Biochem Pharmacol* 1973;22:3099–108.
- Sircar JC, Schwender CF, Johnson EA. Soyabean lipoxygenase inhibition by nonsteroidal anti-inflammatory drugs. *Prostaglandins* 1983;25:393–6.
- Gaffney BJ. Lipoxygenases: structural principles and spectroscopy. *Annu Rev Biophys Biomol Struct* 1996;25:431–59.

41. Mahesha HG, Singh SA, Rao AGA. Inhibition of lipoxygenase by soy isoflavones: evidence of isoflavones as redox inhibitors. *Arch Biochem Biophys* 2007;461:176–85.
42. Harish MS, Nagur M, Badami S. Antihistaminic and mast cell stabilizing activity of *Striga orobanchioides*. *J Ethnopharmacol* 2001;76:197–200.
43. Duan W, Kua IC, Selvarajan S, et al. Anti-inflammatory effects of genistein, a tyrosine kinase inhibitor, on guinea pig model of asthma. *Am J Respir Crit Care Med* 2003;167:185–92.
44. Shore PA, Burkhalter A, Cohn VH. A method for the fluorometric assay of histamine in tissues. *J Pharmacol Exp Ther* 1959;127:182–6.
45. Busse WW, Lemanske JR. Advances in immunology: asthma. *N Engl J Med* 2001;344:350–62.
46. Kips JC. Cytokines in asthma. *Eur Respir J* 2001;18:S24–33.
47. Chung KF, Barnes PJ. Cytokines in asthma. *Thorax* 1999;54:825–57.
48. Mahajan SG, Mehta AA. Role of cytokines in pathophysiology of asthma. *Ir J Pharmacol Ther* 2006;5:1–14.
49. Schellenberg RR, Foster A, Duff MJ. Anti-IgE induced contraction of human bronchus *in vitro*. *J Allergy Clin Immunol* 1985;75:126–30.
50. Phillips GD, Holgate ST. Interaction of inhaled LTC<sub>4</sub> with histamine and PGD<sub>2</sub> on airway caliber in asthma. *J Appl Physiol* 1989;66:304–12.
51. Engineer DM, Morris HR, Piper PJ, Sirois P. The release of prostaglandins and thromboxanes from guinea-pig lung by slow reacting substance, of anaphylaxis, and its inhibition. *Br J Pharmacol* 1978;64:211–18.
52. Haskó G, Linden J, Cronstein B, Pacher P. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat Rev Drug Discov* 2008;7:759–70.
53. Eum SY, Karim M, Hamid Q, et al. Involvement of the cysteinyl-leucotrienes in allergen-induced airways eosinophilia and hyperresponsiveness in the mouse. *Am J Respir Cell Mol Biol* 2003;8:25–32.
54. Mahajan SG, Banerjee A, Chauhan BF, et al. Inhibitory effect of n-butanol fraction of *Moringa oleifera* Lam. seeds on ovalbumin-induced airway inflammation in a guinea pig model of asthma. *Int J Toxicol* 2009;28:519–27.
55. Ngoc LP, Gold DR, Tzianabos AO, et al. Cytokines, allergy, and asthma. *Curr Opin Allergy Clin Immunol* 2005;5:161–6.

Supplementary material available online  
Supplementary data