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The Tyrosine Kinase Inhibitor Masitinib Blunts Airway Inflammation and Improves Associated Lung Mechanics in a Feline Model of Chronic Allergic Asthma

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Key Words
Aeroallergens · Airway eosinophilia · Airway hyperresponsiveness · Asthma model · c-KIT · IgE

Abstract
Background: Blockade of tyrosine kinase signaling by masitinib, a c-kit/PDGF receptor tyrosine kinase inhibitor, can modulate allergic airway inflammation, but effects on lung mechanics have not been well characterized. We hypothesized masitinib would decrease airway eosinophilia and consequently improve pulmonary mechanics in a feline allergic asthma model. ***Methods:*** Asthma was induced in 12 cats using Bermuda grass allergen (BGA). Cats received 50 mg/day oral masitinib or placebo. Bronchoalveolar lavage fluid (BALF) was analyzed for eosinophils, total protein (TP) and BGA-specific IgE. Ventilator-acquired mechanics after methacholine (MCh) challenge determined MCh concentration needed to increase baseline airway resistance by 200% ($EC_{200}R_{aw}$), positive end expiratory occlusion pressure (PEEP) and end inspiratory breath hold pressure (P_{plat}). An inverse correlate of respiratory system compliance P_{plat}-PEEP was also calculated. Data were analyzed using the Wilcoxon test, with one-tailed significance set at $p < 0.1$. ***Results:*** After 4 weeks, percent eosinophils in BALF was lower in masitinib-treated cats (7 ± 9%) versus controls (30 ± 27%, $p = 0.023$). BALF TP significantly differed ($p = 0.047$) between groups, decreasing with masitinib and increasing with placebo. BALF BGA-specific IgE was unaffected by masitinib. Both groups showed an improvement in $EC_{200}R_{aw}$ (masitinib, $p = 0.015$; control, $p = 0.078$) but no significant change in PEEP after 4 weeks. Masitinib-treated cats demonstrated decreased P_{plat} ($p = 0.033$) and P_{plat}-PEEP ($p = 0.075$) at week 4, suggesting an improvement in respiratory compliance. ***Conclusions:*** Masitinib reduced BALF eosinophilia and TP, indicating improved airway inflammation and edema, and improved P_{plat} and P_{plat}-PEEP, suggesting benefit to respiratory compliance influenced by airway inflammation/edema. Masitinib deserves further study in humans with chronic allergic asthma.

Introduction

Tyrosine kinase signaling cascades regulate cell survival, growth, differentiation, and migration of many cell types. Tyrosine kinase inhibitors (TKIs) are small molecule inhibitors that block the ATP-binding site of kinases.

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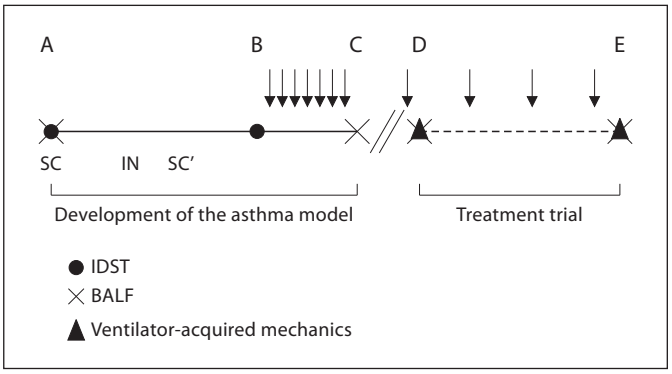

Fig. 1. Timeline of asthma induction and of masitinib/placebo treatment. Healthy research cats were screened for preexisting sensitization to BGA by IDST and for airway inflammation by collection of BALF (A). A subcutaneous injection of BGA in alum and pertussis toxin were administered (SC) on day 0, followed by intranasal BGA (IN) on day 14, and subcutaneous BGA in alum alone (SC′) on day 21. Repeat IDST was performed on day 28 to confirm parenteral sensitization to BGA (B). Seven challenges of BGA by aerosol were delivered over the following 2 weeks, and 24 h after the last aerosol challenge, repeat collection of BALF documented an asthmatic phenotype (C). The treatment trial began with a baseline (D) assessment of BALF and with ventilator-acquired mechanics using MCh as a bronchoprovocant. Aerosol challenges of BGA were continued weekly throughout the treatment trial, including 24 h prior to BALF collection and pulmonary mechanics measurement. Repeat BALF and pulmonary mechanics measurements were performed at 4 weeks (E).

TKIs have a well-documented history of favorable effects in several malignancies [1–4] and inflammatory/fibrotic disorders [5–7].

Allergic asthma is a chronic inflammatory condition of the small airways associated with infiltration of activated mast cells, eosinophils, and T lymphocytes; airflow limitation, and airway remodeling. Stem cell factor (SCF), the growth factor for c-kit receptor, is associated with proliferation and degranulation of mast cells, and development, adhesion, and activation of eosinophils [8–11]. Both SCF and c-kit are present in vivo in the epithelium and subepithelium of human asthmatic airways [12, 13]. Glucocorticoids decrease SCF mRNA expression and mast cell numbers from bronchial mucosal biopsies in human asthmatics, suggesting the SCF/c-kit pathway is a viable target for treatment [13]. This knowledge has led to the investigation of TKIs for the treatment of allergic asthma [14–16].

Cats are the only animal species that spontaneously develop allergic asthma with all the major features of human asthma; thus asthma induction in a species that naturally develops the disease takes advantage of relevant anatomic, physiologic, and immunologic similarities with humans [17–21]. Other advantages of this feline asthma model include maintenance of the asthmatic phenotype chronically without allergen tachyphylaxis and the ability to instrument for pulmonary mechanics and to repeatedly collect bronchoalveolar lavage fluid (BALF) without the need for sacrifice, thus allowing study interventions in the same cat over time.

Masitinib, a relatively new and selective TKI, blocks c-kit, platelet-derived growth factor receptor (PDGFR), Lyn, and, to a lesser extent, fibroblast growth factor receptor 3 [22]. Rigorous assessment of the effect of masitinib on inflammation and dependent pulmonary mechanics in asthma has not been performed to date. We hypothesized that masitinib would decrease airway eosinophilia and, as a result, improve pulmonary mechanics in a preclinical feline model of chronic allergic asthma.

Materials and Methods

Allergen Sensitization, Challenge, and Treatment

Twelve male, domestic shorthair cats were bred from a high-responder asthmatic cat research colony (n = 5; University of Missouri, Columbia, Mo., USA) or acquired commercially (n = 7; Liberty Research, Inc., Waverly, N.Y., USA). Cats were cared for in accordance with the NIH Guide for the Care and Use of Laboratory Animals; the study was approved by the University of Missouri Animal Care and Use Committee. Six cats were used per experimental group.

Cats naïve to Bermuda grass allergen (BGA) underwent allergen sensitization as previously described in detail, with slight modification (fig. 1) [19]. Briefly, on day 0, cats received a subcutaneous injection of 12 μg of BGA (Greer Laboratories, Lenoir, N.C., USA) in 10 mg of alum, and an injection of 100 ng s.c. *Bordetella pertussis* toxin (Sigma Aldrich, St. Louis, Mo., USA); on day 14, 75 μg BGA in 0.2 ml of PBS intranasally, and on day 21, another injection of 12 μg BGA s.c. in 10 mg of alum. On day 28, intradermal skin testing (IDST) confirmed BGA sensitization. Aerosols of BGA (100 μg) in awake, spontaneously breathing cats were administered over the next 2 weeks and BALF collection was used to confirm an asthmatic phenotype (>17% eosinophils).

This was a prospective, blinded, single-center, randomized, placebo-controlled study of daily oral masitinib (50 mg) versus placebo (identical in appearance; provided by AB Science, Paris, France) in experimentally asthmatic cats (dose range 11.1–13.8 mg/kg). BALF and ventilator-acquired mechanics were evaluated at baseline and week 4.

Analysis of BALF: Eosinophils, Total Protein, and IgE

BALF was collected for differential cell counts as previously described [23], centrifuged (300 *g* for 10 min) and supernatant stored at –20°C until analysis. Total protein (TP) was measured

using a modified Bradford protein assay (Bio-Rad Laboratories, Hercules, Calif., USA). BGA-specific IgE was analyzed by ELISA using polyclonal chicken anti-feline IgE antisera and validated similar to previously used polyclonal rabbit antisera [24]. Controls were derived from cats enrolled in an unrelated study. Pooled BALF was obtained prior to induction of asthma (negative control) and after induction of asthma (positive control; confirmed to be IgE reactive by Prausnitz-Kustner testing). Results were expressed as an optical density (OD) and normalized to TP:

$(OD_{sample}/TP_{sample})/(OD_{positive\ control}/TP_{positive\ control})$.

Evaluation of Pulmonary Mechanics

Cats were intubated with a 4-mm cuffed endotracheal tube after induction of anesthesia with propofol (6 mg/kg i.v.). Anesthesia was maintained using propofol at a rate of 0.2 mg/kg/min i.v. Intermittent boluses of propofol (0.5–1 mg/kg i.v.) were administered as needed to maintain an appropriate anesthetic depth and suppress spontaneous breaths. After intubation, cats were ventilated via an Engström Carestation ventilator (GE Healthcare, Fairfield, Conn., USA) equipped with a neonatal flow sensor at the oral end of the endotracheal tube. Ventilation was delivered using a volume-controlled ventilation mode, a tidal volume of 10 ml/kg, at 10 breaths/min, FIO_2 0.4, and an inspiratory-to-expiratory ratio of 1:3. Cats were ventilated for a minimum of 5 min prior to data collection. Airway reactivity was evaluated by obtaining ventilator-calculated airway resistance (R_{aw}) measurements in response to methacholine (MCh) challenge as previously described [25].

Sterile saline (0.9%) was administered by aerosol for 30 s using the Aeroneb Solo in-line nebulizer (Aerogen, Galway, Ireland). Data were then collected for 4 min. End-inspiratory (plateau) pressure (P_{plat}) was measured with a 5-second end-inspiratory breath hold maneuver at min 2 and 4 of data collection. Positive end expiratory occlusion pressure (PEEP) was measured on end-expiratory breath hold at closely adjacent time points. MCh was subsequently delivered in doubling doses (0.0625–32 mg/ml) by aerosol for 30 s with 4 min of data collection between each dose. MCh challenge was terminated when R_{aw} increased 200% above baseline. P_{plat}, PEEP, and P_{plat}-PEEP values, measured at the highest MCh delivered ($EC_{200}R_{aw}$), were compared between treatment groups.

Data from the MCh challenge were expressed as (a) the effective concentration of MCh that increased baseline R_{aw} by 200% ($EC_{200}R_{aw}$); (b) changes in P_{plat}; (c) changes in PEEP, and (d) the P_{plat}-PEEP difference. If $EC_{200}R_{aw}$ was not achieved with 32 mg/ml of MCh, the value was set at 32 mg/ml for analysis. The average post-saline P_{plat} (2 measurements over 4 min) was compared with average post-MCh P_{plat}. The highest dose of MCh administered at week 0 was recorded; post-MCh P_{plat} at week 4 was measured at that same MCh dose. This was done to maintain consistency with the degree of challenge with the bronchoprovocant.

Safety Evaluation

Visual inspection for adverse reactions after daily medication, physical examinations, complete blood counts, serum biochemical profiles, urinalyses, and urine protein:creatinine ratios were obtained throughout the study. Mild biochemical alterations or adverse events resulted in temporary drug interruption until the abnormality resolved, at which time medication was resumed at a dose of 50 mg every other day. Severe adverse events, such as proteinuria, mandated study withdrawal.

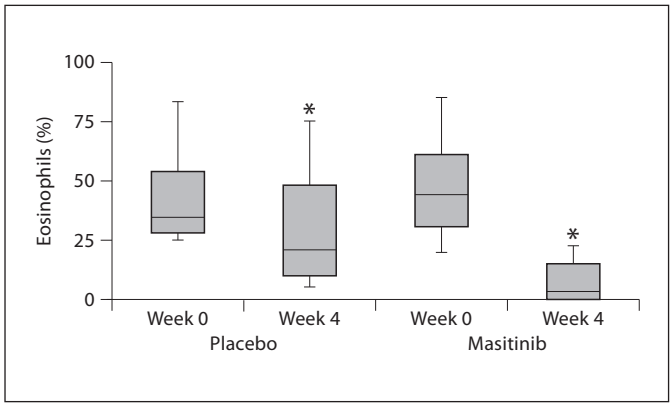

Fig. 2. Effect of masitinib on BALF eosinophilia after allergen challenge. Experimentally asthmatic cats (n = 6/group) were randomized to receive masitinib (50 mg/day) or placebo for 4 weeks. BALF was collected prior to treatment (week 0) and at week 4. In the box-and-whisker plot, the horizontal line represents the median value with the box providing the interquartile range and the whiskers the full range of observations. The percentage of BALF eosinophils was significantly lower in masitinib-treated cats at week 4. * $p = 0.023$ vs. placebo.

Statistical Analysis

A Wilcoxon rank-sum test was performed to compare treatment groups with each other at baseline and week 4 for percentages of BALF eosinophils, BALF TP, BALF IgE, $EC_{200}R_{aw}$, PEEP, P_{plat}, and P_{plat}-PEEP. A Wilcoxon signed rank test was performed to compare the change in the above parameters over 4 weeks within each of the treatment groups (placebo/masitinib). The proportion of adverse events in each group was compared using Fisher's exact test. We used one-tailed tests because we hypothesized that masitinib would have unidirectional effects based upon its mechanism of action. Considering the small study population size, anticipated variability in measurements and objective of demonstrating proof-of-concept, $p < 0.10$ was considered significant (equivalent to a two-tailed $p < 0.05$).

Results

Effects of Masitinib on Markers of Airway Inflammation

There was no significant difference in percent BALF eosinophils between the two groups prior to treatment ($p = 0.345$; fig. 2). After 4 weeks of treatment, the percentage of BALF eosinophils was significantly lower in masitinib- compared with placebo-treated cats ($p = 0.023$; fig. 2). There was no significant difference between groups in the BALF TP at baseline ($p = 0.115$). Change in BALF TP after 4 weeks of treatment was significantly different

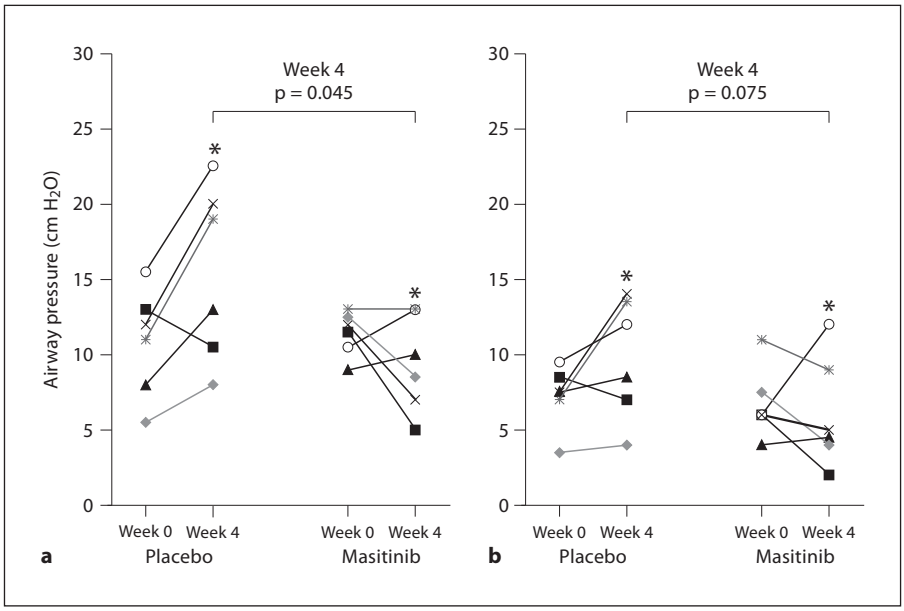

Fig. 3. Effects of masitinib on ventilator-acquired pulmonary mechanics. Each line represents the data from an individual asthmatic cat treated with placebo or masitinib after MCh challenge; P_{plat} and PEEP values were measured at the highest dose of MCh required to induce $EC_{200}R_{aw}$. **a** P_{plat} significantly decreased in masitinib-treated cats after 4 weeks compared with placebo (p = 0.045). **b** P_{plat}-PEEP also significantly differed between treatments at week 4, with a decrease in masitinib-treated cats and an increase in placebo-treated cats (p = 0.075).

between groups (p = 0.047). BALF TP increased (from 0.078 ± 0.027 mg/ml at baseline to 0.141 ± 0.116 mg/ml at week 4) in the placebo group, but decreased (from 0.166 ± 0.120 mg/ml at baseline to 0.103 ± 0.046 mg/ml at week 4) in the masitinib group. No significant difference between treatment groups was noted in BALF BGA-specific IgE at baseline (p = 0.405) and week 4 (p = 0.288), or change from baseline to week 4 (p = 0.288; data not shown).

Effects of Masitinib on Pulmonary Mechanics

No significant difference was found in $EC_{200}R_{aw}$ between masitinib-treated and placebo-treated groups at baseline (5.8 ± 8.8 and 2.4 ± 1.4 mg/ml, respectively; p = 0.405). While the $EC_{200}R_{aw}$ increased from baseline to week 4 in both groups (masitinib 7.6 ± 10.1 mg/ml, p = 0.016; placebo 7.4 ± 10.3 mg/ml, p = 0.078), there was no significant difference between treatment groups at week 4 (p = 0.468).

Breath hold maneuvers, PEEP and P_{plat}, resulted in discordant yet explicable outcomes. PEEP measurements did not differ between groups at baseline (p = 0.233) or week 4 (p = 0.315). At baseline, MCh challenge raised P_{plat} in most cats, reflective of expected airflow limitation in asthmatic cats. Unlike outcomes with PEEP, P_{plat} was significantly lower in the masitinib group at week 4 (masitinib 9.4 ± 3.2 cm H_2O vs. placebo 15.5 ± 5.8 cm H_2O, p = 0.045; fig. 3a). Comparing the change in P_{plat} from baseline to week 4, values differed significantly (p = 0.012), being lower in the masitinib group (–2.0 ± 3.6 cm H_2O) and higher in the placebo group (4.7 ± 4.1 cm H_2O). P_{plat}-PEEP calculations were not different between groups at baseline (masitinib 6.6 ± 2.6 cm H_2O and placebo 7.3 ± 2.0 cm H_2O; p = 0.186). However, at week 4, a significant difference in P_{plat}-PEEP was noted between groups (fig. 3b), with a decrease in the masitinib group compared to an increase in the placebo group (masitinib 5.6 ± 3.9 cm H_2O and placebo 10.4 ± 4.9 cm H_2O; p = 0.075). Pulmonary mechanics of masitinib-treated cats revealed a reduction in P_{plat} and P_{plat}-PEEP, with no change in PEEP or $EC_{200}R_{aw}$.

Safety

The most frequent adverse event noted in association with masitinib administration was moderate-to-severe proteinuria (masitinib 6/6 cats; placebo 0/6 cats; p = 0.002); proteinuria was present without isosthenuria or azotemia. After week 4, proteinuria resulted in temporary masitinib interruption in 2 cats and permanent discontinuation of the drug in 4 cats. In all cats, proteinuria was self-limiting upon discontinuation of masitinib without further intervention. Clinical side effects noted in the masitinib-treated group, represented as total number of episodes out of 269 doses of masitinib administered, were vomiting (n = 4) and diarrhea (n = 1). Self-limiting biochemical abnormalities included hypoalbuminemia, hypercholesterolemia, and increased ALT (n = 1 for each). Cytopenia (neutropenia, anemia, or thrombocytopenia)

was not noted in any cat. Although routine testing revealed transient laboratory abnormalities, the cats had normal physical examinations.

Discussion

This is the first study to show that chronic administration of the c-kit/PDGFR TKI masitinib significantly reduces airway inflammation and improves lung compliance in a model of chronic allergic asthma. Distal airway inflammation (measured in this study by percent BALF eosinophils and TP) has been shown to correlate with edema and consequent reduction in respiratory compliance (inverse correlate of P_{plat}-PEEP) [26], and were blunted with masitinib in our study. Masitinib did not have an effect on MCh-induced changes in $EC_{200}R_{aw}$ [traditional measure of airway hyperreactivity (AHR)] or PEEP, both indicators of *larger* airway constriction and air trapping.

Murine models of asthma using other multitargeted TKIs have suggested benefit in blunting eosinophilic airway inflammation, AHR, and airway remodeling [14, 15], but not compliance. One group found a decrease in both peribronchial eosinophils and peak R_{aw} with imatinib treatment of cockroach allergen-induced asthmatic mice [14]. However, this study only measured AHR after a single dose of imatinib prior to allergen challenge, thereby potentially maximizing the drug effect and evaluating only acute responses to the drug. A second study showed a reduction in eosinophilic inflammation, AHR, and remodeling in ovalbumin-sensitized mice treated with chronic doses of sunitinib, administered simultaneous to the ovalbumin challenges required to induce asthma [15]. Thus, while the *development* of hallmark features of asthma is blunted, the study did not assess the effects of TKI on *established* chronic asthma, which is more relevant to human studies. Additionally, both studies used the allergen-challenged murine model, which has been criticized for lack of biological relevance, leading to few translations from basic discovery to successful clinical application [27]. The cat is the only animal species that commonly and *spontaneously* develops asthma, including the major features of human allergic asthma (airway eosinophilia, AHR, and airway remodeling) [17–21]. Thus, induction of asthma in the cat more closely models natural development and exacerbation of human asthma; additionally, given that they are more outbred than rodents and have intact immune systems, they are better suited for preclinical studies.

This study demonstrates a significant favorable effect of chronic masitinib treatment on small airway inflammation and hyperresponsiveness by way of improved lung compliance. Cats treated with oral masitinib demonstrated lower P_{plat} and a lower P_{plat}-PEEP; measurement techniques in cats were previously characterized in our feline asthma model utilizing the Engström Carestation ventilator [25]. P_{plat}-PEEP reflects lung resistive pressure, an inverse parameter of respiratory system compliance, which often improves with small airway dilation [28] and reduction in small airway edema or inflammation. Discordant effects of masitinib on PEEP, compared with P_{plat} or P_{plat}-PEEP, suggest that the drug confers benefit on compliance but not air trapping. This phenomenon may be a consequence of the effects of masitinib on improved *small* airway inflammation without a demonstrable favorable effect on *large* airways (i.e., no significant change in $EC_{200}R_{aw}$). Previously, a phase 2a study in severe corticosteroid-dependent asthmatics showed that masitinib significantly improved asthma symptoms (assessed by the Asthma Control Questionnaire) [16], despite the lack of a favorable effect on FEV_1. These clinical findings are consistent with and explained by our data. We also demonstrated that masitinib-treated cats experienced improved measures of *small* airway inflammation (decreased the percentage of BALF eosinophils and TP) and obstruction/compliance (decreased P_{plat}-PEEP), despite having no significant improvement in *large* airway resistance/AHR.

This study also evaluated the safety of masitinib in cats. Masitinib blocks c-kit, PDGFR, and Lyn [22], which have differential expression between cells, tissues, and species. The major adverse reaction noted in this study was proteinuria, without renal insufficiency/failure, in cats receiving 50 mg/day of masitinib. In contrast, a prior study on masitinib tolerance in healthy cats reported proteinuria at a lower frequency of 2/10 (20%) cats treated with 50 mg/day masitinib experiencing clinically relevant proteinuria, and none (0/10) when treated at 50 mg every other day [29]. Proteinuria due to minimal-change nephropathy has been previously documented in a dog [30], the pathophysiology of which was not evaluated in our study. Proteinuria in all affected cats resolved upon drug discontinuation, without the need for further intervention. Further characterization of chronic masitinib effects on kidney function is warranted.

In conclusion, chronic masitinib treatment in chronic allergic feline asthma was associated with dampened airway eosinophilia and lung resistive pressure. Masitinib demonstrated a favorable effect on *smaller airway*

inflammation/obstruction and consequential respiratory system compliance, without seemingly playing a role in *large airways*. Masitinib may have a role in the treatment of chronic asthma and maintenance of asthma control.

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