



Pulmonary, Gastrointestinal and Urogenital Pharmacology

Acidinium bromide abrogates allergen-induced hyperresponsiveness and reduces eosinophilia in murine model of airway inflammation

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ABSTRACT

Airway hyperresponsiveness and inflammation characterize the airways of individuals with asthma and chronic obstructive pulmonary disease (COPD). Hence, therapeutic approaches that attenuate such manifestations may offer promise in the management of these diseases. In the present study, we investigated whether a novel long-acting cholinergic antagonist, acidinium bromide, modulates airway function and leukocyte trafficking in an *Aspergillus fumigatus* (Af)-induced murine model of asthma. Nebulized acidinium (1 mg/ml) administration completely abrogated increases in methacholine-induced lung resistance in Af-exposed mice. Parallel assessment of dynamic compliance showed that acidinium also completely restores methacholine-mediated decreases in naïve and Af-exposed mice. As evidenced by differential cell counts within bronchoalveolar lavage fluid, acidinium also diminished (51 ± 4%) Af-induced airway eosinophil numbers with no significant change in other immune cell types. Further assessment of cytokine and total protein levels in bronchoalveolar lavage fluid showed that acidinium had little effect on IL-4 or IL-6 levels in either Af-exposed or naïve mice but markedly decreased total protein levels in bronchoalveolar lavage fluid. These data suggest that acidinium, a selective muscarinic antagonist, not only acts as a bronchodilator but could also act as an anti-inflammatory agent with potential clinical benefits in the treatment of COPD and asthma.

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1. Introduction

Acetylcholine, a pivotal airway parasympathetic neurotransmitter, promotes airway smooth muscle shortening and mucus secretion in airways (Belmonte, 2005). As enhanced parasympathetic activity correlates with airway inflammation and hyperresponsiveness, the use of anticholinergics has been advocated to yield substantial therapeutic benefits in treatment of asthma and COPD. In human studies, the concomitant use of long-acting β_2 adrenergic agonists and long-acting muscarinic receptor antagonists provides clinically relevant improvements in bronchodilation and patient symptoms compared to treatment with either agent alone (Tashkin et al., 2008; van Noord et al., 2006). In addition to restoring airway function, the combination of a long-acting muscarinic receptor antagonist with a long-acting β_2 adrenergic agonist effectively decreases the rate of COPD exacerbations (Cazzola and Matera, 2008). In animal studies, such combinations substantially attenuate bronchoconstriction fol-

lowing a variety of challenges (Rossoni et al., 2007). Interestingly, even parameters independent of airway smooth muscle relaxation such as thromboxane A release were significantly altered by long-acting muscarinic receptor antagonists suggesting broader therapeutic benefits when used in conjunction with existing respiratory medications.

Among the five subtypes of muscarinic receptors (M_1 – M_5), the M_3 receptors are localized in airway smooth muscle tissue and mediate vagal and methacholine-induced bronchoconstrictor responses (Coulson and Fryer, 2003). Accordingly, bronchodilation occurs by selective antagonism of airway localized M_3 receptors, while non-selective antagonism may also promote systemic blockade of M_2 receptors inducing unwarranted cardiovascular effects (Barnes, 2004). Acidinium bromide, an inhaled cholinergic antagonist with sub-nano molar affinity for all muscarinic receptors, manifests a high kinetic selectivity for the M_3 receptor (Gavalda et al., 2009b). In comparison to existing anti-muscarinic drugs including tiotropium, acidinium undergoes rapid hydrolysis in human plasma, resulting in low and transient systemic exposure, diminishing the potential for class-related systemic side effects. In the present study, using a well-characterized murine model of allergen-induced airway hyperresponsiveness and inflammation, we studied the effect of acidinium

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in modulating *Aspergillus fumigatus* (Af)-induced airway hyperresponsiveness. We also investigated the anti-inflammatory potential of acridinium by measuring leukocyte numbers and cytokine levels in bronchoalveolar lavage fluid.

2. Materials and methods

2.1. Animals and sensitization protocol

Eight-12-week-old female BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were housed under pathogen-free conditions. This study was approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Two cohorts of mice were evaluated: “Naïve” mice received intranasal vehicle challenges with 21% glycerol in phosphate-buffered saline (PBS) and “Af-sensitized” mice injected intraperitoneally (i.p.) with 20 µg Af (Bayer Pharmaceuticals, Elkhart, IN) together with 20 mg alum (Imject Alum; Pierce, Rockford, IL) in 100 µl of PBS on Days 1 and 14, followed by intranasal challenge on Days 25, 26, and 27 with 25 µl of Af extract in PBS (12.5 mg in 21% glycerol, PBS) as described previously (Kurup et al., 1992). Af-sensitized and naïve mice were anesthetized by inhalation of isoflurane, and 25 µl of Af extract or vehicle, respectively, was applied to the left naris. The studies were performed and all mice were sacrificed on Day 28, 24 h after their last intranasal treatment.

2.2. Acridinium treatments

Subsets of Af-sensitized (Af+AB) and naïve mice (AB) were treated twice a day with acridinium bromide (AB; 1 mg/ml) for three days (Days 25, 26, and 27) before assessing airway function and lung inflammation (Day 28). On each acridinium treatment session, acridinium was administered as an aerosol by an ultrasonic nebulizer in two 1-minute sessions with a 4-minute interval between each administration.

2.3. Assessment of airway responsiveness to methacholine

Airway responsiveness to methacholine was assessed using total lung resistance measurement by FlexiVent® as described previously (Haczku et al., 2001, 2000; Jain et al., 2008). Lung function tests were assessed 24 h after the Af or saline challenge. Bronchial reactivity to aerosolized methacholine was measured using the FlexiVent® system (SCIREQ, Montreal, Canada). Lung mechanics were studied in tracheostomized mice under anesthesia by intraperitoneal injection of ketamine and xylazine. Mice were ventilated with a computer-controlled small-animal ventilator (SCIREQ) with a rate of 150 breaths/min, and a positive end-expiratory pressure of 2 to 3 cm H₂O. Once ventilated, mice were paralyzed with 0.8 mg/kg pancuronium bromide to block spontaneous breathing. The single-compartment model was fitted to these data by multiple linear regressions to calculate dynamic resistance and compliance of the airway.

2.4. Assessment of total protein, cytokine and immune cell in bronchoalveolar lavage fluid

Lungs were lavaged using 2 ml of sterile saline. The amount of liquid recovered after bronchoalveolar lavage averaged 1.75 ml. Total protein in cell-free bronchoalveolar lavage fluid was assayed by a BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL). Cytokine levels were determined from cell-free supernatant of the bronchoalveolar lavage fluid by ELISA using antibodies and recombinant cytokines from R&D Systems (San Diego, CA). Total and differential cell counts and ELISA analysis were performed as described previously (Kierstein et al., 2008).

2.5. Statistical analysis

To analyze the effect of different doses of methacholine on changes in lung resistance and dynamic compliance in the two groups, we used two-way ANOVA ± Bonferroni adjustment. All values are represented as mean ± S.E.M. Two means were considered significantly different when the P value was <0.05.

3. Results

3.1. Acridinium reduces Af-induced airway hyperresponsiveness

The baseline lung resistance values in all groups (naïve, Af, AB or Af+AB) after saline challenge were not significantly different from each other. As shown in Fig. 1, Af sensitization of mice induced a dose-dependent increase in methacholine-stimulated lung resistance with a peak resistance of 8.8 ± 1.1 cm H₂O s/ml at 20 mg/ml of methacholine. Acridinium administration abrogated Af-induced increases in lung resistance at 5, 10 and 20 mg/ml of methacholine by $66 \pm 4\%$, $82 \pm 2\%$ and $88 \pm 4\%$, respectively. As depicted in Fig. 2, with cumulative doses of methacholine, a dose-dependent decrease in dynamic compliance was also observed in both naïve and Af-treated mice. In naïve and Af-sensitized groups, acridinium administration substantially ($P < 0.05$, ANOVA) reversed methacholine-induced reduction in dynamic compliance. Naïve mice that received intranasal glycerol treatment alone showed no difference compared to non-sensitized, normal BALB/c mice in any of the study's parameters.

3.2. Acridinium attenuates Af-mediated changes in total proteins in bronchoalveolar lavage fluid

Total protein levels within bronchoalveolar lavage were assayed to index Af-induced lung injury and capillary leakage. As evidenced in Fig. 3, Af treatment increased the total amount of protein in bronchoalveolar lavage, and this effect was attenuated by acridinium treatment by $63 \pm 18\%$.

3.3. Acridinium diminishes Af-induced increases in bronchoalveolar lavage fluid-eosinophil numbers

Quantitative and selective increases in bronchoalveolar lavage fluid-resident immune cell populations are important indicators of lung inflammation. Consistent with increased total protein content in bronchoalveolar lavage fluid, allergen sensitization significantly

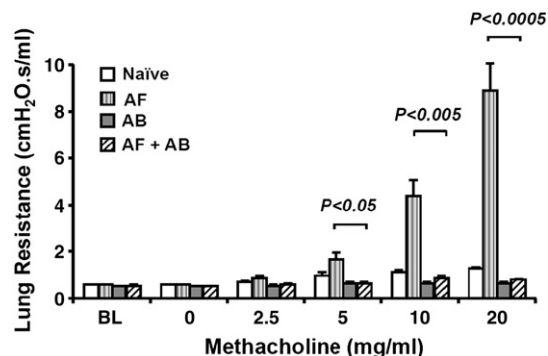


Fig. 1. Acridinium bromide substantially abrogates Af-induced hyperresponsiveness. The effect of varying doses of methacholine (Mch) on lung resistance (cm H₂O s/ml) in naïve and Af-sensitized mice treated with and without aerosolized acridinium bromide (AB) as assessed by measuring total lung resistance by Flexivent. Each group of naïve and Af consisted of 6 mice, and AB and Af+AB groups had 12 mice. Values are mean ± S.E.M.; statistical analysis was performed by two-way ANOVA ± Bonferroni adjustment. $P < 0.05$ was considered to be significantly different.

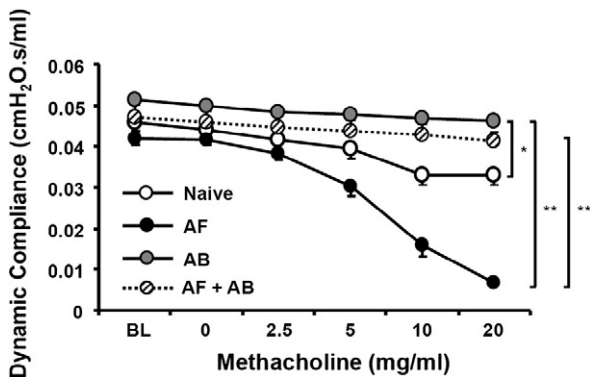


Fig. 2. Acridinium bromide substantially inhibits *Af*-mediated decreases in dynamic compliance. AB treatment of *Af*-sensitized and naïve animals substantially restored dose-dependent decreases in methacholine-induced changes in dynamic compliance. Each cohort of naïve and *Af* consisted of 6 mice, and AB and *Af* + AB groups had 12 mice. Values expressed are mean $\pm$ S.E.M; statistical analysis was performed by two-way ANOVA $\pm$ Bonferroni adjustment. *, Statistically significant at $P < 0.05$; **, statistically different at $P < 0.005$.

enhanced cell numbers over naïve controls. Compared to naïve animals, assessment of constitutive cell populations in *Af*-sensitized groups showed a significant increase ($P < 0.05$, ANOVA) in eosinophil numbers. As shown in Fig. 4, while *Af* treatment showed an eosinophil count of $10 \pm 1.3 \times 10^5$ cells, acridinium treatment substantially diminished *Af*-induced eosinophilia to $4.3 \pm 0.3 \times 10^5$ cells (a decrease of $56 \pm 4\%$) with no significant effects on other immune cell types.

3.4. Acridinium has no effect on *Af*-induced cytokine levels

As shown in Fig. 5, evaluation of bronchoalveolar lavage fluid by ELISA showed that acridinium treatment has no significant effect ($P < 0.05$, ANOVA) in mitigating *Af*-induced increases in either IL-4 (60 ± 19 pg/ml *Af* alone, 82 ± 45 pg/ml *Af* + AB) or IL-6 levels (64 ± 14 pg/ml *Af* alone, 42 ± 12 pg/ml *Af* + AB). Additionally, acridinium had little effect on basal and *Af*-induced increases in TNF and IL-13 levels (data not shown).

4. Discussion

Autoradiographic studies have shown M_3 receptors on airway smooth muscle and epithelial cells of large and small human airways (Mak and Barnes, 1990). Additionally, M_3 receptors are localized to

endothelial cells of the bronchial circulation and can mediate the vasodilator response to cholinergic stimulation (Wess, 2004). Functionally, bronchoconstrictor responses in lung tissue are primarily mediated via M_3 receptors in airway smooth muscle, as confirmed in tissue preparations derived from $M_3R^{-/-}$ knockout mice (Fisher et al., 2004). In this study, aerosolized acridinium substantially inhibited *Af*-induced hyperresponsiveness in mice as evidenced by abrogation of methacholine-induced lung resistance and dynamic compliance. While acridinium displays competitive antagonism by its association with both M_2 and M_3 receptor subtypes, AB dissociates faster from the M_2 receptor than the M_3 receptor, thus conferring a kinetic selectivity for the M_3 subtype (M_3 residence $t_{1/2} = 4 \times M_2$ residence $t_{1/2}$) (Gavalda et al., 2009a). Studies in guinea pigs have shown that acridinium has a fast onset of action ($t_{1/2} = 6.8 \pm 1.5$ min, $t_{max} = 35.9 \pm 8.2$ min) in reverting carbachol-induced tracheal contractions, long duration of action ($t_{1/2} = 29$ h), and to undergo rapid hydrolysis in plasma ($t_{1/2} = 2$ min) thus providing a favorable therapeutic index as a long-acting muscarinic receptor antagonist (Alberti et al., 2009).

Following *Af* challenge, increased airway responsiveness and inflammation coincide with an increased number of eosinophils in the bronchoalveolar lavage fluid, and the depletion of eosinophils abrogates antigen-induced hyperactivity (Hamelmann et al., 1997; Holgate et al., 1991). In our studies, acridinium administration substantially inhibited *Af*-induced airway eosinophilia with no significant effects on the trafficking of other leukocytes. Our results support the conclusion from early studies where M_3 receptor antagonists were shown to substantially diminish ovalbumin (OVA)-induced eosinophil influx in lung tissue (Bos et al., 2007). The precise mechanism by which acridinium diminished *Af*-induced airway eosinophilia in our study remains unknown and could be similar to the role of atropine in inhibiting acetylcholine-mediated airway vascular leakage and plasma extravasations in guinea pig lungs (Cui et al., 2008).

In murine models of airway inflammation, prominent changes in total protein content in bronchoalveolar lavage fluid correlates with increased influx of leukocytes and vascular permeability (Haczku et al., 2001; Kleeberger and Hudak, 1992; Papouchado et al., 2001). As illustrated in Fig. 3, acridinium treatment abrogated *Af*-induced increases in total protein content. Whether anticholinergics modulate airway responses by controlling eosinophil numbers or activation is unclear. While diminishing antigen-induced eosinophil numbers in sensitized guinea pigs, atropine pre-treatment potentiated M_2 receptor-independent vagal hyperactivity and enhanced eosinophil major basic protein presence. These studies imply that

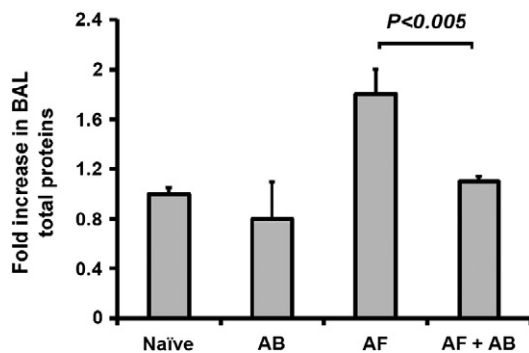


Fig. 3. Acridinium bromide diminishes *Af*-induced increases in bronchoalveolar lavage fluid total protein. Proteins in bronchoalveolar lavage fluid were measured using the Bradford protein assay with a standard curve for albumin. Each group of naïve and *Af* consisted of 6 mice, and AB and *Af* + AB groups had 12 mice. The data are expressed as mean $\pm$ S.E.M proportional increase over naïve controls. Statistical analysis was performed by two-way ANOVA $\pm$ Bonferroni adjustment. $P < 0.05$ was considered to be significantly different.

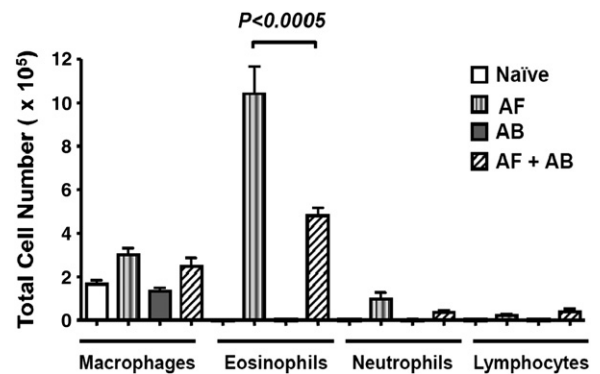


Fig. 4. Acridinium bromide substantially abrogates *Af*-induced airway eosinophilia. Bronchoalveolar lavage fluid analysis of *Af*-sensitized and naïve animals treated with and without AB for macrophages, eosinophils, neutrophils, and lymphocytes. Data in the figure is representative of mean cell counts $\pm$ S.E.M from 6 animals each in AB and *Af* + AB groups and 12 animals each in naïve and *Af* groups. Statistical analysis was performed by two-way ANOVA $\pm$ Bonferroni adjustment. $P < 0.05$ was considered to be significantly different.

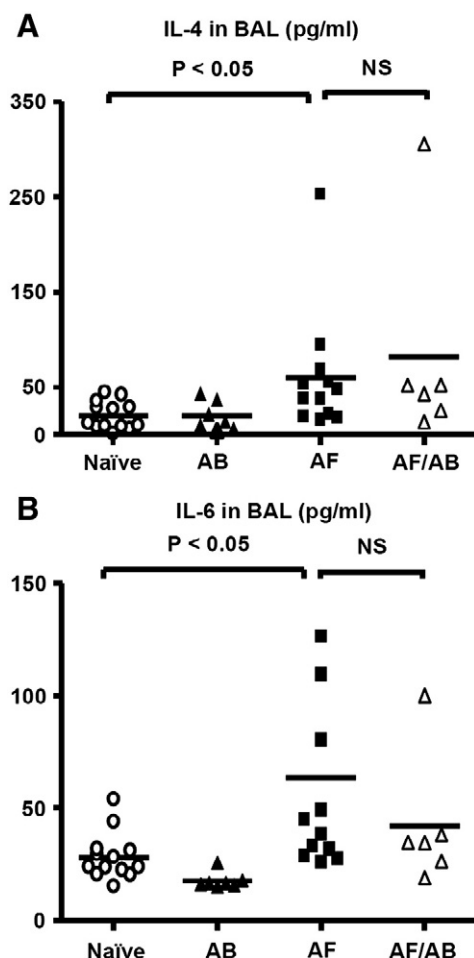


Fig. 5. Acridinium bromide does not alter *Af*-mediated cytokine levels in bronchoalveolar lavage fluid. Bronchoalveolar lavage fluid obtained from each mouse was assessed in triplicate for IL-4 (A) and IL-6 (B) by ELISA. AB treatment had no effect on either IL-4 or IL-6 levels in naïve or *Af*-sensitized animals. Data represents group mean $\pm$ S.E.M. Each cohort of naïve and *Af* consisted of 6 mice, and AB and *Af*+AB groups had 12 mice. Statistical analysis was performed by ANOVA $\pm$ Bonferroni adjustment. $P < 0.05$ was considered to be significantly different.

anticholinergics can regulate airway responses by modulating eosinophil activation (Verbout et al., 2007). Earlier reports also suggest that cholinergic agonists affect airway obstruction by modulating mucin secretion, smooth muscle proliferation and levels of inflammatory mediators (Cui et al., 2008; Gosens et al., 2006). Recent studies showed that acetylcholine-mediated activation of M_3 receptors enhance chemotactic factors that mediate leukocyte retention in airways (Profita et al., 2005). Similarly, stimulation of bovine tracheal strips with carbachol resulted in increased expression of IL-8, cyclooxygenase-1 and -2 at the mRNA level (Kanefsky et al., 2006). Plausibly, antagonism of M_3 receptors by acridinium could have diminished *Af*-induced eosinophil numbers in bronchoalveolar lavage fluid as seen in our studies. Acridinium could diminish mast cell-mediated bronchoconstriction by antagonizing mACh receptors in parasympathetic ganglia as reported by others (Cyphert et al., 2009). Accordingly, inhibition of mast cell degranulation may also diminish histamine dependent increases in vascular permeability. The current data suggest that acridinium attenuates *Af*-induced increases in total protein levels in bronchoalveolar lavage fluid, consistent with an effect on vascular permeability. The aforementioned mechanisms remain speculative and warrant further investigation.

IL-4 is a key cytokine in allergic inflammation secreted by varied cell types in the airway wall and bronchoalveolar lavage fluid. IL-4 orchestrates migration and residence of immune cells to inflamma-

tory loci by induction of adhesion molecules in the pulmonary vasculature (Steinke and Borish, 2001). Earlier studies showed that in mice treated with anti-IL-4 antibody during allergen sensitization or the airway challenge of IL-4-deficient mice, both airway eosinophilia and airway hyperresponsiveness are modulated by the cytokine (Corry et al., 1996; Renz et al., 1995). Multiple external stimuli, including OVA, *Af* and ozone, enhance IL-6 secretions in murine airways. While disruption of IL-6-mediated responses by IL-6 α -blocking antibody diminishes OVA-induced Th2 inflammation, others have shown that *Af*-mediated mucus secretions are substantially diminished in IL-6 $^{-/-}$ mice, suggesting a prominent role for IL-6 in mediating/resolving an asthmatic phenotype (Doganci et al., 2005; Neveu et al., 2009). Evidence now shows that acetylcholine, its synthesizing enzyme choline acetyltransferase, and its functional receptors present in most airway resident cells and leukocytes can modulate physiological outcomes, including cytokine secretions, implying that M_3 agonism or antagonism can regulate inflammatory mechanisms (Blanchet et al., 2007; Kanefsky et al., 2006; Klapproth et al., 1998; Racke and Matthiesen, 2004; Wessler and Kirkpatrick, 2001). However, acridinium, in our studies, had little effect on *Af*-mediated IL-4 or IL-6 levels in bronchoalveolar lavage, implying that IL-4/IL-6-independent mechanisms likely modulate eosinophil localization and airway hyperresponsiveness. Our unexpected finding is consistent with other studies showing that airway hyperresponsiveness is regulated independent of airway inflammation and eosinophilia in animals deficient in IL-4 α chain or in animals lacking signal transducer and activator of transcription (STAT)-6 (Kumar and Foster, 2002) or with studies where comparison of IL-6 $^{-/-}$ or wild type mice showed no substantial differences in *Af*-induced airway eosinophils (Neveu et al., 2009).

Previously, clinical studies have shown that acridinium improves lung function in patients with COPD. Our studies here show a potential therapeutic benefit for acridinium bromide beyond a direct effect on bronchodilation in effectively abrogating airway hyperresponsiveness and airway eosinophilia in allergen-induced airway hyperresponsiveness. These studies complement growing evidence for the benefits of long-acting anticholinergic agents as bronchodilators and potential anti-inflammatory agents in the treatment of asthma and COPD. Based on our studies, evaluating the role of acridinium in mediating M_3 receptor-driven outcomes in isolated human airway tissues and exploring the mechanisms by which acridinium inhibits eosinophil accumulation in lungs would further delineate the mechanisms by which long-acting muscarinic receptor antagonists reduce bronchoconstriction and airway inflammation in asthma and COPD.

Conflict of interest

Jose Freire is an employee of Forest Research Institute, which supported this study. The authors alone are responsible for the content and writing of the paper.

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References

- Alberti, J., Martinet, A., Sentellas, S., Salva, M., 2009. Enzymatic hydrolysis of acridinium bromide in human plasma by human esterases. *Am. J. Respir. Crit. Care Med.* 179.
- Barnes, P.J., 2004. The role of anticholinergics in chronic obstructive pulmonary disease. *Am. J. Med.* 117 (Suppl 12A), 24S–32S.

- Belmonte, K.E., 2005. Cholinergic pathways in the lungs and anticholinergic therapy for chronic obstructive pulmonary disease. *Proc. Am. Thorac. Soc.* 2, 297–304 discussion 311–292.
- Blanchet, M.R., Langlois, A., Israel-Assayag, E., Beaulieu, M.J., Ferland, C., Laviolette, M., Cormier, Y., 2007. Modulation of eosinophil activation in vitro by a nicotinic receptor agonist. *J. Leukoc. Biol.* 81, 1245–1251.
- Bos, I.S., Gosens, R., Zuidhof, A.B., Schaafsma, D., Halayko, A.J., Meurs, H., Zaagsma, J., 2007. Inhibition of allergen-induced airway remodelling by tiotropium and budesonide: a comparison. *Eur. Respir. J.* 30, 653–661.
- Cazzola, M., Matera, M.G., 2008. Novel long-acting bronchodilators for COPD and asthma. *Br. J. Pharmacol.* 155, 291–299.
- Corry, D.B., Folkesson, H.G., Warnock, M.L., Erle, D.J., Matthay, M.A., Wiener-Kronish, J. P., Locksley, R.M., 1996. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *J. Exp. Med.* 183, 109–117.
- Coulson, F.R., Fryer, A.D., 2003. Muscarinic acetylcholine receptors and airway diseases. *Pharmacol. Ther.* 98, 59–69.
- Cui, Y.Y., Zhu, L., Wang, H., Advenier, C., Chen, H.Z., Devillier, P., 2008. Muscarinic receptors involved in airway vascular leakage induced by experimental gastro-oesophageal reflux. *Life Sci.* 82, 949–955.
- Cyphert, J.M., Kovarova, M., Allen, I.C., Hartney, J.M., Murphy, D.L., Wess, J., Koller, B.H., 2009. Cooperation between mast cells and neurons is essential for antigen-mediated bronchoconstriction. *J. Immunol.* 182, 7430–7439.
- Doganci, A., Eigenbrod, T., Krug, N., De Sanctis, G.T., Hausding, M., Erpenbeck, V.J., Haddad el, B., Lehr, H.A., Schmitt, E., Bopp, T., Kallen, K.J., Herz, U., Schmitt, S., Luft, C., Hecht, O., Hohlfeld, J.M., Ito, H., Nishimoto, N., Yoshizaki, K., Kishimoto, T., Rose-John, S., Renz, H., Neurath, M.F., Galle, P.R., Finotto, S., 2005. The IL-6R alpha chain controls lung CD4+CD25+ Treg development and function during allergic airway inflammation in vivo. *J. Clin. Invest.* 115, 313–325.
- Fisher, J.T., Vincent, S.G., Gomez, J., Yamada, M., Wess, J., 2004. Loss of vagally mediated bradycardia and bronchoconstriction in mice lacking M2 or M3 muscarinic acetylcholine receptors. *FASEB J.* 18, 711–713.
- Gavalda, A., Calama, E., Gomez-Angelats, M., Carcasona, C., Llenas, J., Beleta, J., Miralpeix, M., 2009a. In vitro functional profile of acridinium bromide in isolated guinea pig trachea and left atria. *Am. J. Respir. Crit. Care Med.* 179.
- Gavalda, A., Miralpeix, M., Ramos, I., Otal, R., Carreno, C., Vinals, M., Domenech, T., Carcasona, C., Reyes, B., Vilella, D., Gras, J., Cortijo, J., Morcillo, E., Llenas, J., Ryder, H., Beleta, J., 2009b. Characterization of acridinium bromide, a novel inhaled muscarinic antagonist, with long duration of action and a favorable pharmacological profile. *J. Pharmacol. Exp. Ther.* 331, 740–751.
- Gosens, R., Zaagsma, J., Meurs, H., Halayko, A.J., 2006. Muscarinic receptor signaling in the pathophysiology of asthma and COPD. *Respir. Res.* 7, 73.
- Haczku, A., Atochina, E.N., Tomer, Y., Chen, H., Scanlon, S.T., Russo, S., Xu, J., Panettieri Jr., R.A., Beers, M.F., 2001. *Aspergillus fumigatus*-induced allergic airway inflammation alters surfactant homeostasis and lung function in BALB/c mice. *Am. J. Respir. Cell Mol. Biol.* 25, 45–50.
- Haczku, A., Takeda, K., Hamelmann, E., Loader, J., Joetham, A., Redai, I., Irvin, C.G., Lee, J.J., Kikutani, H., Conrad, D., Gelfand, E.W., 2000. CD23 exhibits negative regulatory effects on allergic sensitization and airway hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* 161, 952–960.
- Hamelmann, E., Oshiba, A., Loader, J., Larsen, G.L., Gleich, G., Lee, J., Gelfand, E.W., 1997. Anti-interleukin-5 antibody prevents airway hyperresponsiveness in a murine model of airway sensitization. *Am. J. Respir. Crit. Care Med.* 155, 819–825.
- Holgate, S.T., Roche, W.R., Church, M.K., 1991. The role of the eosinophil in asthma. *Am. Rev. Respir. Dis.* 143, S66–S70.
- Jain, D., Keslacy, S., Tliba, O., Cao, Y., Kierstein, S., Amin, K., Panettieri Jr., R.A., Haczku, A., Amrani, Y., 2008. Essential role of IFN β and CD38 in TNF α -induced airway smooth muscle hyper-responsiveness. *Immunobiology* 213, 499–509.
- Kanefsky, J., Lenburg, M., Hai, C.M., 2006. Cholinergic receptor and cyclic stretch-mediated inflammatory gene expression in intact ASM. *Am. J. Respir. Cell Mol. Biol.* 34, 417–425.
- Kierstein, S., Krytska, K., Sharma, S., Amrani, Y., Salmon, M., Panettieri Jr., R.A., Zangrilli, J., Haczku, A., 2008. Ozone inhalation induces exacerbation of eosinophilic airway inflammation and hyperresponsiveness in allergen-sensitized mice. *Allergy* 63, 438–446.
- Klapproth, H., Racke, K., Wessler, I., 1998. Acetylcholine and nicotine stimulate the release of granulocyte-macrophage colony stimulating factor from cultured human bronchial epithelial cells. *Naunyn-Schmiedeberg Arch. Pharmacol.* 357, 472–475.
- Kleeberger, S.R., Hudak, B.B., 1992. Acute ozone-induced change in airway permeability: role of infiltrating leukocytes. *J. Appl. Physiol.* 72, 670–676.
- Kumar, R.K., Foster, P.S., 2002. Modeling allergic asthma in mice: pitfalls and opportunities. *Am. J. Respir. Cell Mol. Biol.* 27, 267–272.
- Kurup, V.P., Mauze, S., Choi, H., Seymour, B.W., Coffman, R.L., 1992. A murine model of allergic bronchopulmonary aspergillosis with elevated eosinophils and IgE. *J. Immunol.* 148, 3783–3788.
- Mak, J.C., Barnes, P.J., 1990. Autoradiographic visualization of muscarinic receptor subtypes in human and guinea pig lung. *Am. Rev. Respir. Dis.* 141, 1559–1568.
- Neveu, W.A., Allard, J.B., Dienz, O., Wargo, M.J., Ciliberto, G., Whittaker, L.A., Rincon, M., 2009. IL-6 is required for airway mucus production induced by inhaled fungal allergens. *J. Immunol.* 183, 1732–1738.
- Papouchado, B.G., Chapoval, S.P., Marietta, E.V., Weiler, C.R., David, C.S., 2001. Cockroach allergen-induced eosinophilic airway inflammation in HLA-DQ/human CD4(+) transgenic mice. *J. Immunol.* 167, 4627–4634.
- Profita, M., Giorgi, R.D., Sala, A., Bonanno, A., Riccobono, L., Mirabella, F., Gjomarkaj, M., Bonsignore, G., Bousquet, J., Vignola, A.M., 2005. Muscarinic receptors, leukotriene B4 production and neutrophilic inflammation in COPD patients. *Allergy* 60, 1361–1369.
- Racke, K., Matthies, S., 2004. The airway cholinergic system: physiology and pharmacology. *Pulm. Pharmacol. Ther.* 17, 181–198.
- Renz, H., Enssle, K., Lauffer, L., Kurrle, R., Gelfand, E.W., 1995. Inhibition of allergen-induced IgE and IgG1 production by soluble IL-4 receptor. *Int. Arch. Allergy Immunol.* 106, 46–54.
- Rossoni, G., Manfredi, B., Razzetti, R., Civelli, M., Berti, F., 2007. Positive interaction of the novel beta2-agonist carmoterol and tiotropium bromide in the control of airway changes induced by different challenges in guinea-pigs. *Pulm. Pharmacol. Ther.* 20, 250–257.
- Steinke, J.W., Borish, L., 2001. Th2 cytokines and asthma. Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists. *Respir. Res.* 2, 66–70.
- Tashkin, D.P., Littner, M., Andrews, C.P., Tomlinson, L., Rinehart, M., Denis-Mize, K., 2008. Concomitant treatment with nebulized formoterol and tiotropium in subjects with COPD: a placebo-controlled trial. *Respir. Med.* 102, 479–487.
- van Noord, J.A., Aumann, J.L., Janssens, E., Verhaert, J., Smeets, J.J., Mueller, A., Cornelissen, P.J., 2006. Effects of tiotropium with and without formoterol on airflow obstruction and resting hyperinflation in patients with COPD. *Chest* 129, 509–517.
- Verbout, N.G., Lorton, J.K., Jacoby, D.B., Fryer, A.D., 2007. Atropine pretreatment enhances airway hyperreactivity in antigen-challenged guinea pigs through an eosinophil-dependent mechanism. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 292, L1126–L1135.
- Wess, J., 2004. Muscarinic acetylcholine receptor knockout mice: novel phenotypes and clinical implications. *Annu. Rev. Pharmacol. Toxicol.* 44, 423–450.
- Wessler, I.K., Kirkpatrick, C.J., 2001. The non-neuronal cholinergic system: an emerging drug target in the airways. *Pulm. Pharmacol. Ther.* 14, 423–434.