Diplomarbeit

NOVEL THERAPEUTICS FOR THE TREATMENT OF EXPERIMENTAL ALLERGIC ASTHMA

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MARIE LE BRAS
0207498
Anthropologie

ao.Prof.Dr. Oskar Hoffmann

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Abstract

Asthma is a chronic lung disease triggered by genetic and environmental risk factors of an individual or a population. Intricate interactions account for a heterogeneous disease profile, and the challenge consist in finding new therapeutic strategies for severe asthma patients, who respond poorly to glucocorticoids, as well as for mild to moderate asthma patients, whose quality of life is considerably reduced by long-term side effects. The aim of this study was to test two different compounds in a mouse model of allergic asthma. Experimental models of acute and relapsing asthma were used to determine the effect of these compounds. Selected key parameters of allergic disease were analyzed.

Our results indicate that one of the drugs tested, Ba 679 BR, is highly effective in preventing airway hyperresponsiveness, but had no effect on acute lung inflammation and mucus production during the onset of allergic asthma. However, this compound at a 1 mg/kg dose had a significant effect on immunological memory responses, illustrated by a reduction of airway inflammation and in particular eosinophilia. The second compound tested, MAM-06.301, was able to reduce mucus hypersecretion in peripheral airways and parenchymal inflammation without altering the inflammatory response in the airways, as measured in the bronchoalveolar lavage fluid.

Taken together, these data illustrate that both compounds modulate important features of allergic asthma in experimental models. We would argue that the Ba 679 BR compound may be useful in the treatment of exacerbations of allergic asthma and that MAM-06.301 may be effective as an adjuvant therapy for allergic asthma but it is necessary to further investigate the usefulness of these compounds as potential adjuvants and steroid-sparing therapeutics.

Unsere Ergebnisse sagen aus, dass eines der getesteten Medikamente, Ba 679 BR, während der Krankheitsentstehung, vorbeugend gegen eine Hyperreaktivität der Luftwege wirkt. Allerdings konnte eine Wirkung von Ba 679 BR weder auf die Entzündung, noch auf die Schleimproduktion der Atemwege nachgewiesen werden. Bei einer Dosis von 1 mg/kg, weist das Medikament jedoch einen signifikanten Effekt auf die immunologische Gedächtnisantwort auf, gezeichnet durch eine Reduktion der Atemwegsentzündung, hier insbesondere der Eosinophilen. Das zweite getestete Medikament, MAM-06.301, konnte erfolgreich sowohl die Schleimproduktion der peripheren Lunge als auch die parenchymale Entzündung reduzieren. Die entzündliche Antwort in den Luftwegen wurde nicht beeinflusst, wie aus der Analyse des Bronchoalveolären Lavages hervorging.

Contents

1 Introduction .................................................. 1
  1.1 The socioeconomic impact of asthma .................. 1
  1.2 Medical definition and etiology of asthma .......... 2
    1.2.1 Definition and classification of asthma .......... 2
    1.2.2 Etiology of asthma ............................... 3
  1.3 Aim ....................................................... 3
  1.4 Pathological features of asthma ...................... 4
    1.4.1 Airway inflammation in allergic asthma: inflammatory cells and mediators .......................... 4
    1.4.2 AHR .................................................. 8
    1.4.3 Airway remodeling ................................... 8
  1.5 Asthma medication ....................................... 9
    1.5.1 Current asthma treatments ......................... 9
    1.5.2 Therapeutic issues, need and challenges for the development of novel therapeutics .................. 10
    1.5.3 Improvement of current therapies ................ 11
    1.5.4 Mediator antagonists .............................. 12
    1.5.5 Novel anti-inflammatory treatments ............... 13
  1.6 Two novel compounds for the treatment of asthma .... 14
    1.6.1 Tiotropium Bromide (Spiriva, Ba 679 BR) ........ 14
    1.6.2 Escin ............................................... 17
  1.7 Mouse model of experimental allergic asthma ......... 20

2 Materials and Methods ..................................... 23
  2.1 Experimental design of the in vivo model ............ 23
    2.1.1 Mice ............................................... 23
    2.1.2 Setup of experimental allergic asthma and treatment protocol in the C57BL/6 mouse strain .......... 23
2.1.3 Setup of experimental allergic asthma, Ba 679 BR treatment protocol and measurement of AHR in the BALB/c mouse strain

2.2 Processing of bronchoalveolar fluid, blood and lung tissue after the assessment

2.2.1 Serum collection

2.2.2 Collection of bronchoalveolar lavage fluid (BALF)

2.2.3 Preparation of BALF slides by cytospin centrifugation

2.2.4 Analysis of leukocyte subsets by differential cell count of BALF monolayer after Wright-Giemsa staining

2.2.5 Fixation of the lungs and embedding in paraffin

2.3 Histopathological staining methods

2.3.1 Deparaffinazation, dehydration and rehydration of the lung tissue

2.3.2 Periodic acid - Schiff staining of lung sections for mucus producing goblet cells

2.3.3 LUNA staining of lung sections for the presence of eosinophils in infiltrates

2.3.4 Haematoxylin and eosin staining of lung section for cell infiltration

2.4 Detection of milk - specific IgG1 in the serum by ELISA

2.5 Detection of inflammatory cytokines in BALF supernatant by ELISA

2.6 Statistical analysis

3 Results

3.1 Effect of Tiotropium Bromide on an acute model of allergic asthma

3.1.1 Effect of Tiotropium Bromide treatment on AHR induced in an acute model of allergic asthma

3.1.2 First experimental approach of the acute model of experimental asthma

3.1.3 Second experimental approach of the acute model of experimental asthma

3.1.4 Third experimental approach of the acute model of experimental asthma

3.2 Effect of Tiotropium Bromide on a memory model of allergic asthma

3.2.1 Effect of Tiotropium Bromide treatment on inflammatory cells in BALF
3.2.2 Effect of Tiotropium Bromide treatment on inflammation in lung tissue .............................................. 56
3.2.3 Effect of Tiotropium Bromide treatment on allergen-specific IgG1 antibodies .............................................. 56
3.3 Effect of MAM-06.301 on an acute model of allergic asthma ................. 62
  3.3.1 First experimental approach of the acute model of experimental asthma ....................................................... 62
  3.3.2 Second experimental approach of the acute model of experimental asthma ...................................................... 68
  3.3.3 Third experimental approach ......................................................... 75

4 Discussion ................................................ 81
  4.1 Experimental mouse model of milk-induced allergic asthma ................. 81
    4.1.1 Usefulness of our mouse model .............................................. 81
    4.1.2 Effect on allergic airway inflammation ..................................... 82
  4.2 New insights on the effect of the novel compounds on inflammation processes ................................................... 83
    4.2.1 Investigations on a new indication for Tiotropium Bromide ........ 83
    4.2.2 The natural component MAM-06.301 for a new application ......... 85
  4.3 Summary ....................................................... 87

A Acronyms ........................................... I

List of Figures ........................................ III

B Bibliography ........................................ V

B Curriculum Vitae ..................................... XXIII
Chapter 1

Introduction

1.1 The socioeconomic impact of asthma

Asthma is a major global public health problem with increasing incidence and prevalence. According to the World Health Organization (WHO), 300 million people from all ages and ethnic backgrounds suffer from asthma worldwide, a total that is expected to rise to about 400 million over the next 15-20 years. Asthma is still responsible for about one out of 250 deaths worldwide and 80% of asthma deaths occur in low and lower-middle income countries (WHO). Worldwide, asthma accounts for around 1% of all DALYs lost worldwide, which reflects the high prevalence and severity of the disease.

No prevention or cure exist for asthma yet, but in most patients, asthma can be controlled with existing therapies. However, 5-10% of the asthma cases can only be poorly controlled and are associated with serious impairment of the patient’s quality of life as well as considerable consequences for the health care system. In fact, these patients account for more than 50% of the total health care costs associated with asthma, due to frequent emergency room visits and hospitalization (direct costs) as well as related work and school absenteeism (indirect costs). Taken together, the high socioeconomic burden of the disease relies to a large extent on the consequences of uncontrolled illness course, hence an important factor which drives the active research on novel therapeutics for asthma.

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1. Generally, the impact of a disease in the society can be measured in terms of loss of disability-adjusted life years (DALYs), where one DALY is one year of healthy life lost due to premature death or disability.
Nevertheless, apart from the necessity to better understand the mechanisms underlying asthma, there is also an urgent need to find new therapeutical approaches in order to ameliorate the conditions of asthma patients with controlled asthma.

**1.2 Medical definition and etiology of asthma**

**1.2.1 Definition and classification of asthma**

Asthma is a chronic lung disease that is defined by its clinical, physiological and pathological features. Clinically, patients have recurrent episodes of coughing, wheezing, breathlessness and chest tightness, particularly at night or in the early morning, suggesting a circadian rhythm of symptoms emergence. Physiologically, asthma is characterized by airway hyperresponsiveness (AHR) in response to a stimulus that would be innocuous in healthy individuals, which leads to expiratory airflow limitation caused by reversible airways obstruction. Airway obstruction in asthma is the consequence of bronchoconstriction, bronchial edema and increased mucus production. The dominant pathological feature is airway inflammation resulting from the recruitment of inflammatory cells as well as many mediators, and associated with airway structural changes, called airway remodeling. Structural changes include goblet cell hyperplasia, associated with increased mucus production in the airways, airway smooth muscle hypertrophy and hyperplasia, along with subepithelial fibrosis.

The classification of symptoms severity is useful for the management of the disease, e.g. for the decision about an adequate treatment, at the initial diagnosis of the patient. Asthma is subdivided in four main categories: intermittent, mild persistent, moderate persistent and severe persistent asthma. The attribution of patients to a certain group is decided according to frequency of symptoms during day and at night, airflow limitation quantified by FEV₁ (Forced Expiratory Volume in 1 second) expressed as a percentage of the Vital Capacity or PEV (Peak Expiratory Flow) and lung function variability expressed by FEV₁ or PEV variability.
1.2.2 Etiology of asthma

The strongest risk factors for developing asthma result from complex interactions between genetic predisposition and environment of an individual or a population. In a broader context, genetic predisposition to atopy or AHR belong to the main host factors. Thus, others like obesity and sex were found to influence the expression of asthma. Several environmental factors such as indoor allergens (e.g. house dust mites, furred animals, molds, yeasts, fungi), outdoor allergens (e.g. pollens, molds...), tobacco smoke, chemical irritants, occupational sensitizers, air pollution and even food lead to allergic sensitization and are triggers of asthma development in susceptible organisms. One of the most disputed area concerns the influence of viral infections on the development of allergic asthma. Whereas the “hygiene hypothesis” suggests that a lack of early childhood exposure to infectious agents, symbiotic microorganisms (e.g. gut flora), and parasites increase susceptibility to allergic diseases, including asthma, by modulating immune system development, it is at the same time evident that infections are classical triggers of asthma exacerbations. Taken all together, the incredible complexity of asthma is the interplay of single genetic or environmental factors, dependent or independent from each other, which accumulate and result in unique patterns of etiology in patients or group of patients. The etiology of asthma can therefore not be precisely determined due to the striking heterogeneity among the risk factors.

1.3 Aim

The objective of my work was to test the effectiveness of two compounds in an experimental mouse model of allergic asthma. Despite the high-effective current treatment available for most of the asthma patients, development of novel drugs against asthma stays indispensable for different reasons. A treatment is fully effective, only if the disease for which it has been indicated can be clearly diagnosed and if patients adhere consequently to the dosage protocol. Asthma is a heterogeneous

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2 Atopy: the propensity of an individual to produce IgE antibodies in response to various environmental antigens and to develop strong allergic responses

3 Occupational asthma: term used when disease development is specially associated with the work environment of the patient, e.g. “baker’s asthma”
Chapter 1 Introduction

disease and the treatment needs to be adjusted to the patients’ need in a continuous manner, in order to assure the best possible control and management of the disease. This has led to the implementation of guidelines in the health system as reported by the “Global Initiative for asthma” of 2007\textsuperscript{15}. A safe and easy strategy to ensure a good compliance is the introduction of long-lasting drugs that can reduce the daily intake frequency and thus augment adherence to the treatment and the time between asthma exacerbations. An adequate treatment against asthma should involve the control of its underlying inflammation processes, as well as the relief of bronchospasms. First of all, it is necessary to present the key players in asthma mechanisms and the current medication, to subsequently understand the problematic, the needs and the challenges in therapy development. Finally, I will present the two compounds I tested and emphasize their promising role in the contribution to a better control of asthma by novel classes of drugs.

1.4 Pathological features of asthma

1.4.1 Airway inflammation in allergic asthma: inflammatory cells and mediators

Airway inflammation in asthma involves several inflammatory cells and multiple mediators that result in characteristic pathophysiological changes and is strongly associated with AHR and symptoms\textsuperscript{27,136}. The airway inflammation is persistent even though symptoms are episodic, and the relationship between the severity of the disease and the intensity of inflammation is not clearly established yet\textsuperscript{22,37}. The inflammatory processes start before the appearance of asthmatic symptoms with the infiltration of a normally innocuous antigen (Ag) through the epithelium, as this tissue represents the first defense barrier between environment and body. At the site of inflammation, the inhaled allergens encounter antigen-presenting cells (APCs) - dendritic cells (DCs) and macrophages - and mast cells.

DCs

Immature DCs are present in the epithelial of the respiratory tract and control the lung environment. They capture encountered Ags by endocytosis and transport these
1.4 Pathological features of asthma

to the draining lymph nodes. During their migration, DCs mature to become very efficient at presenting Ag and stimulating naive T cells by constitutive expression of costimulatory molecules and MHC class II molecule. Therefore, DCs are crucial in sensitization to allergens, have the capacity to initiate primary immune responses (priming) and new evidences suggest a role in maintenance of established inflammation.\textsuperscript{67,91} In the lung, DC subsets have the ability to induce different types of immune responses. Thus, Ag presentation by myeloid DCs leads to Th2 sensitization typical of allergic disease, whereas antigen presentation by plasmacytoid DCs serves to dampen inflammation.\textsuperscript{67,92} It has been also shown, that the lineage commitment of naive CD4+ T cells into their functionally different subtypes (discussed later in this section) is guided by activated toll-like receptors (TLRs) present on the surface of DCs and polarized by inhaled antigens.\textsuperscript{17,74}

**Macrophages**

Macrophages are efficient in phagocytosis of large Ags and are activated by CD4+ T cells. They are derived from circulating monocytes, which migrate to the lungs in response to chemoattractant signals.

**Mast cells**

Mast cells display the high-affinity FcεRI receptors on their surface, which bind the Fc portion of IgE antibodies (Abs). When Ags bind to IgE, cross-linking of these Abs occur, thus activating mast cells to synthesize and release several bronchoconstrictors, including histamine and the lipid mediators leukotriene C4, leukotriene D4, leukotriene E4 and prostaglandin D2. Epithelial cells on the surface of the airway express the stem-cell factor (SCF) or c-kit ligand) and contribute to recruit mucosal mast cells which express the c-kit receptor on their surface. Mast cells also release cytokines linked to allergic inflammation, including IL-4, IL-5 and IL-13 and their presence in airway smooth muscle has been linked to AHR.\textsuperscript{25}

**T cells**

In allergic and more specifically in asthmatic patients, the transcription factor GATA3 (GATA-binding protein 3) is activated in naive CD4+ T cells leading to a lineage
commitment and differentiation into T helper 2 (Th2) type of CD4+ T cells. These cells in turn secrete Th2-type cytokines. IL-4 and IL-13 drive on one hand the IgE production in B cells, on the other hand the binding of IL-4 to its receptor leads to the activation of transcription factor STAT6 which in turn regulates the downstream signal for GATA3 activation and contributes therefore to sustain the secretion of Th2 cytokines during inflammation. IL-5 is responsible for eosinophil differentiation in the bone marrow and IL-9 attracts and drives the differentiation of mast cells. IL-13 induces mucus hypersecretion and AHR. The transcription factor Tbet is crucial for Th1 commitment and secretion of Th1 type cytokine IFN-γ, but regulates Th2 cytokines by inhibiting the function of GATA3. The T cell specific transcription factors NFAT (nuclear factor of activated T cells) also seems to enhance the transcriptional activation of GATA3 by targeting the IL4 promoter. The newly discovered IL-33 (member of IL-1 family of cytokines) appears to promote Th2-cell differentiation by translocating to the nucleus and regulating transcription by epigenetic mechanisms. By binding to the surface receptor ST2 of Th2 cells, IL-33 also acts as a chemoattractant of Th2 cells.

Regulatory T cells (Tregs), another subtype of CD4+ T cells, have a crucial role in the maintenance of peripheral tolerance by exhibiting suppressive effects on other CD4+ T cells. A subset of these cells is characterized by expression of CD25 and transcription factor FOXP3 and is currently subject of numerous studies. They may have a role in asthma by regulating Th2 cell function but the mechanisms of regulation seem to vary among allergic airway diseases, leading to unsharp and non-generalizable conclusions.

The Th17 subset of CD4+ T cells has been shown to have an important role in inflammatory and autoimmune diseases\textsuperscript{134,143}. Their generation involves IL-23 and IL-21, as well as the transcription factor RORγt and STAT3 and even if its role in asthma remains unclear, interesting observations have been reported\textsuperscript{113}. Increased concentration of IL-17 (predominant cytokine produced by Th17) have been reported in the sputum of asthmatic patients\textsuperscript{26}, and IL-17 has been associated with neutrophilic inflammation, playing a role in certain phenotypes of asthma\textsuperscript{99,106,132}. Like mentioned above, Th17 cells also produce IL-21, which acts as a positive autoregulatory mechanism by stabilizing Th17 differentiation and as a negative regulatory mechanism by inhibiting FOXP4 expression and thus Tregs development. IL-22 is released as well and stimulates the production of the anti-inflammatory cytokine IL-10.
It is also uncertain, which role play invariant natural killer T cells (iNKT cells) in asthma. This new subset of T cells is CD1-restricted\textsuperscript{4}, presents characteristics of both natural killer cells (NK cells) and conventional T cells and expresses an invariant $\alpha$-chain of their T cell receptor (TCR). A population of iNKT cells exhibits a Th2-like phenotype as they secrete IL-4 and IL-13 and were shown to induce AHR in a mouse model of asthma\textsuperscript{107,138}.

CD8+ T cells are present in patients with more severe disease and irreversible airway obstruction\textsuperscript{139}.

\textbf{B cells}

The cytokines IL-4 and IL-13 induce B cells to undergo immunoglobulin isotype class switching to produce allergen-specific IgE. The IgE antibodies can then bind to its high-affinity Fc receptors (Fc$\varepsilon$RI) expressed on the surface of mast cells and basophils or to its low-affinity Fc receptors (Fc$\varepsilon$RII) expressed by other inflammatory cells, including B cells, macrophages and possibly eosinophils\textsuperscript{62}.

\textbf{Granulocytes}

Eosinophils are present in increased numbers in the airways of most asthmatic patients. During inflammation, epithelial cells release the chemokine ligand 11 (CCL11) also known as eotaxin-1, which stimulates eosinophil recruitment to the lung through the chemokine receptor 3 (CCR3) expressed on their surface. IL-5 produced by Th2 cells is critical for differentiation of eosinophils and for sustaining eosinophilic inflammation. The functional role of eosinophils in asthma is not clear though, as its role in participation of AHR has been questioned after the disappointing results obtained by blocking of IL-5, that did not reduce AHR but only eosinophilic inflammation\textsuperscript{93}. Eosinophils are suggested to have a role in airway fibrosis and interestingly, their presence seems to be a good marker for prediction of corticosteroid sensitivity.

Neutrophilic inflammation has been associated with a non-eosinophilic phenotype of asthma seen in patients with severe asthma, but their role remains uncertain.

\textsuperscript{4}CD1 loads glycolipids and not protein antigens.
1.4.2 AHR

AHR is an important hallmark of asthma and is linked to inflammation and airway remodeling. The contractibility of airways smooth muscle is increased and leads to the typical airway narrowing and associated symptoms in response to special trigger. Furthermore, sensory nerves may be sensitized by inflammation leading thus to exaggerated bronchoconstriction.

1.4.3 Airway remodeling

The airways of asthmatic patients are characterized by the presence of inflammatory cells, but also by structural changes including mucus metaplasia, smooth muscle hyperplasia, subepithelial fibrosis and increased angiogenesis. Airway remodeling is probably driven by mediators released as a consequence of chronic allergic inflammation\textsuperscript{116}. It is hypothesized that the origin of asthma lies in the epithelium and not in primarily in inflammatory pathways. Susceptibility genes expressed by the epithelial have been identified, decreasing the resistance of the airway to environmental stimuli\textsuperscript{75}. Subepithelial fibrosis is present in all asthmatic patients even before the appearance of symptoms and results from the deposition of collagen fibers and proteoglycans under the basement membrane. The increased thickness of airway wall is due to airway smooth muscle hypertrophy and hyperplasia driven by inflammatory mediators, and accounts for changes in the mechanical properties of the airways. It seems that angiogenesis further supports the thickening of the airways. Mucus hypersecretion results from goblet cell metaplasia in the epithelium as well as from an increased size of submucosal glands. In transgenic studies, it was demonstrated that the cytokines IL-11 and IL-13 produce responses in the murine airway with features similar to those in human asthmatic tissues. IL-11 caused airway fibrosis with the enhanced accumulation of interstitial collagen, myocytes, and myofibroblasts. IL-13 caused goblet cells metaplasia, enhanced mucin gene expression, enhanced tissue hyaluronic acid accumulation, and subepithelial fibrosis.
1.5 Asthma medication

Medications to treat asthma can be classified as controllers and relievers. Controllers are medications taken daily on a long-term basis to keep asthma under clinical control through their anti-inflammatory effects. Relievers act quickly to reverse bronchoconstriction and relieve its symptoms. They are meant to be applied when needed, and an increasing use of relievers is an indication of disease worsening.

1.5.1 Current asthma treatments

Approaches to treat asthma target bronchoconstriction and airway inflammation, which is best obtained with a combination therapy of glucocorticoids (budenoside or fluticasone) with $\beta_2$-adrenoceptor agonists (formoterol or salmeterol) in a single inhaler. This is currently the most effective anti-asthmatic treatment available and it controls asthma in about 90-95% of patients\textsuperscript{28}; however, 5-10% have severe disease that responds poorly\textsuperscript{1}. The effectiveness of low to moderate doses of inhaled glucocorticoids (GCs) is improved by combination with long-acting $\beta_2$-adrenoceptor agonists (LABA) and is better than that obtained with higher doses of inhaled GCs alone\textsuperscript{35;58}. There is increasing evidence that these two compounds act in a synergistic way, by reciprocal positive interactions, although the mechanisms are not fully understood. Long-term treatment with inhaled GCs reverses airflow obstruction, reduces exacerbations and the need for hospitalization, improves quality of life and may have contributed to decrease asthma-linked deaths. However, long-term use of high-dose inhaled GCs is problematic as it has serious side effects, such as cataracts, osteoporosis in elderly patients, and stunting of growth in children\textsuperscript{1}. The combination therapy enables therefore a safer treatment by the use of low to moderate dose of GCs and is effective as both maintenance and reliever therapy.

A considerable number of mediators are involved in the complex inflammatory processes occurring in asthma and antagonists against many of these have been developed. Nevertheless, the often disappointing results indicate that inflammation cannot be reduced by the blockade of too specific targets. However, the only mediator antagonists used in asthma therapy are antileucotrienes (or leukotriene modifiers), which either block cysteinyl-leucotriene (cys-LT) receptors (montelukast) or the synthesis of leucotriene (zileuton)\textsuperscript{11;19}. Introduced in the 1990’s, they were the first new class of asthma therapy for over 30 years. Although cys-LT antagonists have
some beneficial clinical effects in asthmatic patients, they have proved to be relatively
weak compared to inhaled GCs. However, they have been shown to be useful as an
add-on therapy to inhaled GCs with the advantage of oral administration without
significant side effects.

It is nevertheless important to note that the current therapies are not curative, as
asthma symptoms and inflammation recur after treatment discontinuation.

1.5.2 Therapeutic issues, need and challenges for the
development of novel therapeutics

The necessity for new therapies consists in two major unmet needs. The first is
an improved compliance in the mild-to-moderate asthma patients, which could be
achieved by the development of safe oral versions of conventional treatments or of new,
more efficacious treatments, ideally pointing towards a cure. The second are patients
with poor controlled severe asthma, which do not respond well to combination therapy,
often due to a GC insensitivity. It became urgent to understand the mechanisms
behind severe asthma in order to identify new targets for drug development and reduce
the uncontrolled asthma cases. Some research areas concentrate for the moment
on a strategy to reverse GC resistance in patient with severe asthma. Furthermore,
the therapeutic effects of GCs are often accompanied by severe and sometimes
irreversible side effects. For this reason, another aspect of research has for goal
development of synthetic GCs, which show reduced side-effects while maintaining
their anti-inflammatory properties[126]. There are more and more evidences, that the
heterogeneity observed in the response to treatment can be attributed to the existence
of different asthma phenotypes caused by different pattern of inflammation[24;66;71;145].

Behind the challenges to face in resolving the therapeutic issues discussed here, is
the need to develop discriminatory handprints of distinct disease subtypes and to
adapt therapies to the heterogeneous and complex aspects of asthma[1;2].
1.5 Asthma medication

1.5.3 Improvement of current therapies

**New longer lasting bronchodilators**

The best reliever therapies existing at the moment for preventing and reversing bronchoconstriction in asthma are the LABA, salmeterol and formoterol, which are effective for 12 hours and additionally increase the duration of action of GCs when both are used in combination. Currently, longer-acting β₂ agonists, the so-called ultra-LABA (ULABA), with a duration of action exceeding the 24 hours are under clinical development and might be suitable for a once-daily dosing. The major advantage of the combination of inhaled GCs with once-a-day, fast onset β₂ agonists, is believed to bring improvement in compliance, rather than a better efficacy of the treatment itself\textsuperscript{103}. Novel class of bronchodilators, such as vasoactive intestinal peptides analogues and K⁺-channel openers, are less adapted due to their vasodilators effect\textsuperscript{13}.

**New corticosteroids with reduced systemic side effects**

The systemic side-effects of inhaled GCs are attributed to their absorption by the lung and are greater with higher GC dose. Efforts are therefore made for the development of safer GCs with low systemic bioavailability, high pulmonary deposition and inactivation or clearance in the circulation. Ciclesonide for example, is a pro-drug activated by lung-specific esterases, which seems to have less systemic effects than currently available GCs. GCs affect gene expression by two mechanisms. The anti-inflammatory effects are mainly mediated by inhibition of transcription factors through a non-genomic effect called transrepression, while transactivation accounts for many of the undesirable side effects and is mediated by genomic effects and binding of GC receptors to DNA. An ensuing interesting approach is the development of dissociated GCs, which are able to selectively activate the transrepression rather than the transactivation mechanism through the GC receptor\textsuperscript{32;126} and are under clinical development.
1.5.4 Mediator antagonists

Beside leukotriene antagonists, inhibitors of pro-inflammatory mediators, like kinins, platelet-activating factor, histamine and prostaglandins (PGs) have proved no beneficial clinical effect against asthma. Efforts are made to find new targets among lipid mediators, but progresses are slow, due to safety and efficacy concerns or to discrepancy between results obtained in animal models and in humans.

Cytokine inhibitors and anti-inflammatory cytokines

There has been a lot of energy investigated in identifying cytokines as targets for new asthma therapies, because of their key role in inflammation and airway remodeling. The demonstration that inhibition of Th2-derived cytokines in many animal models of asthma prevented all aspects of disease drove to the development of antagonists and antibodies directed against IL-4 and IL-5. Deception was great, as these findings could not be replicated in asthmatic patients. However, there is currently interest in blocking IL-13, which is found in higher proportion than IL-4 in asthmatic airways. Other cytokine inhibitors found interest in actual research and I would like to mention the most promising ones. Tumor necrosis factor-α (TNF-alpha) was found to have a role in severe asthma and anti-TNF-α therapies (etanercept and infliximab) are in clinical development. SCF is a key regulator of mast-cell survival in the airways and acts through the c-kit receptor on mast cells. Promising results were obtained in animal models by blockade of SCF or c-kit (imatinib). Thymic stromal lymphopoietin (TSLP) is expressed on airway epithelial cells of asthmatic patients and through its association with dendritic cells activation, became object of intense research in the last years. It was even characterized as a master switch in allergic inflammation and offers new perspectives in therapeutic approach.

Although the use of anti-inflammatory cytokines like IL-10 and IL-12 offered attractive therapeutic perspectives, results were rather disappointing with difficulties to replicate positive effects in humans and undesirable side-effects.

Chemokine and chemokine receptor antagonists

Recruitment of inflammatory cells into the airway is linked by chemokines, small peptides indispensable in the development of asthma, which bind to their respective
1.5 Asthma medication

chemokine receptors. Currently, targeting CCR3 has been of major interest. It is expressed on eosinophils, mast cells and some Th2 cells and its ligands are increased in asthma and mediate eosinophil recruitment. Inhibitors of CCR3 were effective in animal models and are undergoing clinical trials.

1.5.5 Novel anti-inflammatory treatments

Phosphodiesterase inhibitors

Phosphodiesterases (PDE) are found in a variety of inflammatory and structural cells and are classified in 11 families. Most advanced research has been done on the PDE4 inhibitors, which specifically prevent the hydrolysis of cAMP, leading to inhibition of inflammatory cells acting in asthma. Even if these inhibitors (roflumilast and cilomilast) showed high efficacy in animal models, a major limitation in human application is the unattractive side-effect profile, including nausea, vomiting, headaches and gastrointestinal disturbance. Finding inhibitors that would target specific isoenzymes of PDE4, not mediating these side-effects, are now being considered.

Inhibition of transcription factors, kinases and adhesion molecules

The transcription factor nuclear factor-κB (NFκB) plays a key role in the regulation of inflammatory genes involved in asthma pathogenesis as its activation is enhanced in mild asthma and further increases in severe asthma. Interestingly it seems that the NFκB pathway is implicated in GC-resistant asthma. Inhibitors of NFκB undergo preclinical testing. The p38 mitogen activated protein (MAP) kinase activates inflammatory genes in asthmatic processes, and inhibition by an anti-sense molecule was able to suppress pulmonary inflammation in a murine model. As these molecules play a role in a broad range of cell types and of mechanisms, there are concerns about potential dangerous side-effects in humans so that inhalation might be the preferred route of delivery. Among many of the adhesion molecules having a role in asthma, the very late antigen-4 (VLA-4), involved in recruitment of eosinophils and T cells, has been targeted with most success. Currently, inhibitors of VLA-4 are tested in humans, but concerns raise about their long-term safety.
Chapter 1 Introduction

Restoration of GC responsiveness

Data indicate that the GC-receptor mediated suppression of NFκB involves the recruitment of the histone deacetylase-2 (HDAC-2) and that this enzyme activity is decreased in GC-insensitive asthma\(^{12;82;85}\). It has been shown, that low-dose theophylline exerts an anti-asthma effect through increasing activation of HDAC-2, which is subsequently recruited by corticosteroids to suppress inflammatory genes\(^{38;84}\).

Immunomodulation and anti-allergy treatments

There is growing evidence for the central role of IgE in airway inflammation and asthma and the clinical effectiveness of blocking IgE with omalizumab, a recombinant humanized monoclonal antibody directed to the high-affinity IgE receptor (FcεRI) binding domain of the human IgE. It is now approved for asthma therapy in some countries, but present applicability issues\(^{10}\). As the implementation of such a therapy is very cost effective, it can only be used for a small group of patients, with very severe disease\(^{10}\). Additionally, the subcutaneous route of administration presents practical hurdles for patients. However, omalizumab is successful and encourages to further search for inhibitors of IgE-mediated signal transduction pathways. Lumiliximab, an inhibitor of CD23, the low-affinity IgE receptor (FcεRIII), is now being tested in clinical trials.

1.6 Two novel compounds for the treatment of asthma

1.6.1 Tiotropium Bromide (Spiriva, Ba 679 BR)

Drug properties

Tiotropium Bromide is a quaternary ammonium derivative and a structural analog of atropine\(^6\). Tiotropium Bromide is a specific antagonist of muscarinic receptors, which binds to all receptor subtypes with similar affinities, as indicated by similar dissociation constants from the receptors (Kd of M\(_1\), M\(_2\) and M\(_3\) are between 0.01
1.6 Two novel compounds for the treatment of asthma

and 0.04). However, kinetic studies on the half-life of dissociation ($t_{1/2}$) showed that it dissociates very slowly from $M_1$- and $M_3$-receptors ($t_{1/2} = 14.6$ h vs 34.7 h) but more rapidly from $M_2$-receptors ($t_{1/2} = 3.6$ h), thereby giving it a unique kinetic selectivity $^6, ^43$.

![Figure 1.1 Structure of Tiotropium Bromide (C$_{19}$H$_{22}$NO$_4$S$_2$Br.H$_2$O)](image)

**Current indication**

In January 2004, the orally inhaled long-acting anticholinergic bronchodilator Tiotropium Bromide, was approved by the US FDA for the once-daily maintenance treatment of patients with chronic obstructive pulmonary disease (COPD). COPD is characterized by a progressive development of not fully reversible airflow limitation, and represents one of the most common chronic diseases. COPD is largely caused by smoking, although other factors exist, such as air pollution. Tiotropium demonstrates a significantly longer duration of protection than an equipotent dose of its predecessor ipratropium, most likely due to its slow dissociation from human $M_3$-receptors $^43, ^69$. It is a quaternary ammonium derivative which results in little systemic absorption following inhalation and thereby in little side-effects.

Since its introduction on the market, data support the use of tiotropium once-daily as first-line maintenance treatment in patients with COPD $^{141}$. Tiotropium is effective in improving dyspnea, exacerbations and related hospitalizations, health-related quality of life and lung function in patients. It exceeds the benefits seen with ipratropium $^{141}$ or with LABA treatment (salmeterol) $^{45}$. Moreover, anticholinergics are particularly important bronchodilators in COPD, because the vagal tone appears to be the only reversible component of airflow limitation in this disease $^{122}$. Preliminary data suggest
that combining Tiotropium with LABA may produce additional bronchodilator action in COPD\textsuperscript{137;153}. However, long-term studies are required to clarify Tiotropium’s role in comparison to, or in combination with LABA and to assess its effectiveness in mild and very severe COPD\textsuperscript{14}. Though, Tiotropium treatment seems to be associated with an increase in annual costs per patient, as calculated in two 1-year randomized, double-blind clinical trials in the Netherlands and Belgium\textsuperscript{114}.

COPD and asthma have different diagnostic criteria and treatment paradigms but can occur in the same patient. In a recent study, the spirometric effects of Tiotropium in COPD patients with concomitant asthma were investigated. Satisfying results were obtained, as spirometric improvements along with symptomatic benefit were achieved, as seen by reduced need for rescue medication\textsuperscript{100}.

**Promising facts for a beneficial effect on asthma**

In the last two decades, anticholinergic agents have been generally regarded as the first-choice bronchodilator therapy in the routine management of stable COPD, but only to a lesser extent in asthma.

Acetylcholine (ACh) is the primary parasympathetic neurotransmitter in the airways, associated with the induction of airway smooth muscle contraction and mucus secretion. Parasympathetic activity is increased in airway inflammation, which is the basis for the use of anticholinergic therapy in asthma and COPD\textsuperscript{60}. Additionally new evidences indicated that ACh, acting through muscarinic receptors, had an essential regulatory role in allergen-induced airway smooth muscle remodeling\textsuperscript{59–61}. New insights in muscarinic ACh receptors (mAChR) signaling identified mAChR-regulated pathways as potential novel targets for the treatment of various diseases, notably asthma and COPD\textsuperscript{60;146}. M\textsubscript{1}-receptors in parasympathetic ganglia facilitate cholinergic neurotransmission and therefore enhance cholinergic bronchoconstriction, whereas M\textsubscript{3}-receptors on airway smooth muscle cells and glands mediate bronchoconstriction and mucus secretion. M\textsubscript{2}-receptors at cholinergic nerve endings inhibit the release of ACh and therefore act as feedback inhibitory receptors (autoreceptors)\textsuperscript{6}. Studies investigated the effect of the anticholinergic drug glycopyrrolate in human airways and compared it with ipratropium and later with Tiotropium. Tiotropium treatment assured the longest bronchodilation duration\textsuperscript{63;68;140}. Because of its selectivity for the muscarinic M\textsubscript{3}-receptor, we assumed that Tiotropium protects against bronchoconstriction and aberrant mucus production in asthma.
1.6 Two novel compounds for the treatment of asthma

1.6.2 Escin

Drug properties

The triterpene saponin escin is the active component of the extract of seeds of the horse chestnut tree (*Aesculus hippocastanum*), used for its therapeutic properties. The β but not the α molecular structure of escin is active and available in pharmaceutical products.

![Escin Structure](image)

**Figure 1.2** Structure of escin Ia (C_{50}H_{86}O_{24})

Escin owes its therapeutic application mostly to its anti-edematous, anti-inflammatory and venotonic properties. A wide range of mechanisms underlying these effects is currently known and will be discussed here.

The ability of escin to induce venous contraction is based on its permeabilizing effect on smooth muscle cells. Increase in permeability leads to a higher membrane sensitivity to calcium ions and finally to a calcium-induced, calmodulin-dependent, contraction of these smooth muscles. Furthermore, it has been shown that escin makes the cell membrane mainly permeable to high-molecular weight molecules. The anti-exudative effect of escin has been associated with an increased production of Fα-type PGs leading to vasoconstriction.
Chapter 1 Introduction

The anti-edematous effect of escin has been demonstrated in various studies. In fact, in inflammatory conditions as well as during blood stasis resulting in decreased oxygen supply, a reduction in ATP may occur. An ATP decrease results in a cascade of metabolic events like release of PGs and PAF (platelet activating factor), neutrophil recruitment, adherence and activation, all leading to venous stasis and edema in the case of varicose disease. A protective effect of escin on endothelial cells was shown in *in vitro* models of hypoxia and inflammation. Escin was able to inhibit important steps of the activation of endothelial cells caused by hypoxia, including the decrease in ATP content, which is the starting point of the activation cascade, and the increase in the activity of phospholipase A2, an enzyme responsible for the release of precursors of inflammatory mediators. Escin could prevent the increased neutrophil adherence occurring in hypoxia-activated endothelial cells. This effect was shown to be mediated by an inhibitory action on VCAM-1 (vascular cell adhesion molecule 1) and PECAM-1 (platelet endothelial cell adhesion molecule 1), giving an additional protection on endothelial cell morphology. Additionally, escin pre-treatment reduced IL-6 release from LPS-activated vascular endothelium. Furthermore, the endothelium-protectant effect of escin was confirmed as it was found to be able to enhance the endothelium-dependent relaxation induced by ACh. This effect was attributed to enhanced nitric oxide (NO) production by endothelial NO synthase, a calcium-dependent enzyme, activated by the escin-induced increase of endothelial cell calcium permeability. NO, a potent vasodilator produced by endothelial cells, is thought to be the endothelium-dependent relaxing factor (EDRF) which mediates vascular relaxation in response to ACh, bradykinin and substance P in many vascular beds. NO has been implicated in the regulation of blood pressure and regional blood flow, affects vascular smooth-muscle proliferation and inhibits platelet aggregation and leukocyte adhesion. Taken together, escin reduces neutrophil adherence and activation as well as the release of inflammatory mediators, resulting in the protection of veins and reduction of edema. In a study performed on a model of traumatic brain injury in rats, escin could dramatically inhibit NF-κB activation as well as the expression of TNF-α protein and alleviate rat brain edema.

The anti-inflammatory action of escin is closely connected to its anti-edematous and venotonic properties. It has been shown that escin could inhibit acute inflammation and granuloma formation, cause acceleration of gastrointestinal transit, help recover intestinal motility, and attenuate the formation of postoperative adhesions. Further studies shed light on possible mechanisms underlying an escin-mediated release of NO.
1.6 Two novel compounds for the treatment of asthma

and PGs. An antagonist effect of escin on serotonin (5-HT)\textsuperscript{53,104} and histamine\textsuperscript{120} was supported by studies reporting an acceleration of gastrointestinal transit in mice. Proposed mechanisms are the selective activation of 5-HT2 receptors, which in turn causes the release of NO and PGs and the antagonism of the pro-inflammatory 5-HT1 receptor\textsuperscript{104}. Escin’s anti-inflammatory properties have also been associated with anti-hyaluronidase activity in tissue\textsuperscript{52}. Hyaluronidase hydrolyzes glycosaminoglycans (GAGs), including hyaluronan, in the extracellular matrix during tissue remodeling and its activity increases in chronic inflammatory conditions\textsuperscript{23}. Escin may contributes consequently to shift the balance between synthesis and degradation of proteoglycans\textsuperscript{5} towards net synthesis, strengthening the capillary wall and preventing from leakage\textsuperscript{133}. Anti-obesity effects of escin were also reported. In this context, an effect on escin on pancreatic lipase activity, a fat-cleaving enzyme, was proposed\textsuperscript{78}.

Interesting work has been published in the last two years, indicating possible inhibitory effects of escin on mechanisms associated with cancer development. Thus, escin inhibited colonic aberrant crypt foci formation in rats and regulated the cell cycle growth by inducing p21(waf1/cip1) in \textit{in vitro} cultured colon cancer cells. This novel feature suggests escin as a potential useful candidate for colon cancer chemoprevention and treatment\textsuperscript{117}. Furthermore, a recent study claimed that escin was a potent natural inhibitor of proliferation and inducer of apoptosis in human chronic myeloid leukemia K562 cells \textit{in vitro}, being thus a candidate to be considered for the exploration of potential antileukemia drugs\textsuperscript{112}. New findings declare an effect of escin on endothelial cells proliferation, migration and apoptosis and indicate a potential anti-angiogenic activity via its direct effects on endothelial cells\textsuperscript{142}. According to the results of the study, this effect would be mediated on one hand by an increased expression of thrombospondin-1 (TSP-1), on the other hand by a decreased expression of PKC-\textalpha\textsubscript{C} as well as a decreased activation of p44/42 MAPK (ERK) and p38 MAPK.

**Current indication**

Escin is currently indicated as a safe and effective treatment for chronic venous insufficiency (CVI)\textsuperscript{42,119,120,133}. It is used as well in the prevention of post-operative edema, against hemorrhoids\textsuperscript{133} and a cosmetic use due to anti-aging properties have also been reported\textsuperscript{55,148}. Recently, escin combined with another compound, was found to be successful for the treatment of inner ear perfusion disturbances\textsuperscript{130}.

\textsuperscript{5}Proteoglycans: also called mucopolysaccharides, are formed by linkage of GAGs to core proteins,
Chapter 1 Introduction

Promising facts for a beneficial effect on asthma

The anti-inflammatory and anti-edematous properties of escin make it an attractive candidate for the treatment of allergic inflammation in asthmatic airways. Escin acts on different targets of the inflammation process, that have been shown to have a role in asthma. Escin was recently shown to inhibit NF-κB, a transcription factor with important role in the regulation of inflammatory genes involved in asthma pathogenesis. In fact, in research fields aiming to find new targets for asthma therapy, anti-NFκB molecules are currently being tested. Furthermore, the anti-hyaluronase activity of escin might be of particular interest, as there is enhanced hyaluronic acid accumulation in lung tissue of asthmatic patients. It has been shown that TNF-α and IL-1β, cytokines implicated in the pathophysiology of asthma, were potent enhancers of hyaluronidase expression and activity in human airway epithelial cells and lung fibroblasts. Additionally, as the lipid mediator PAF has been associated with catabolic events occurring in varicose disease and key features of asthma, a putative inhibiting effect of MAM-06.301 on PAF might help reducing airway edema, eosinophil accumulation in the airway wall and AHR. Taken together, escin has beneficial effects on several aspects of inflammation pathways, thus being a promising candidate for the reduction of airway inflammation and remodeling in asthma.

1.7 Mouse model of experimental allergic asthma

The application of generalizable mouse models in the modeling of human diseases became an indispensable tool in medical research and contributes to evident progress in understanding pathological processes and developing treatment strategies. An innovative model of experimental allergic asthma has been established in our laboratory consisting in using low-fat powder milk as a natural and clinically relevant allergen for the sensitization of C57BL/6 mice. Whereas the latter showed considerable disease pattern, BALB/c mice turned out to be unresponsive for this protocol. Milk generates an allergic response to a combination of proteins and mimics therefore allergic asthma in patients with the significant advantage of being inexpensive. The pathological picture obtained showed evidences of a Th2-driven allergen-induced pulmonary inflammation and confirmed findings with other allergens, like ovalbumin in BALB/c mice. The mouse model of allergic asthma shares many features with
human asthma, including the development of eosinophilic inflammation in lung tissue and in the bronchoalveolar lavage fluid, Ag-specific Ab response (IgG1 and IgE), and the production of Th2 cytokines (IL-4). The remitting and relapsing process induced by antigenic stimulation can be mimicked in a mouse model of experimental allergic asthma and consequently be used advantageously to test the therapeutic effects of the compounds at different stages of the disease.
Chapter 2

Materials and Methods

2.1 Experimental design of the in vivo model

2.1.1 Mice

C57BL/6 and BALB/c female mice were purchased from Charles River laboratories and used when they were 6 to 8 weeks old. They were maintained in the facilities of the pharmacology department and the veterinary medicine. OVA-free food and water ad libitum were given to the mice. All experimental protocols were complied with the requirements of the Austrian Ministry of Science.

2.1.2 Setup of experimental allergic asthma and treatment protocol in the C57BL/6 mouse strain

Experimental approach for Tiotropium bromide (Ba 679 BR) treatment in the acute model of experimental allergic asthma

Mice were sensitized and challenged with low-fat powder milk. They were immunized 10 µg milk in 200 µl 1x PBS intraperitoneally on days 0 and 21. The mice were exposed to aerosolized milk solution dissolved in 1x PBS, produced by an ultrasonic nebulizer, following three different protocols. Either twice a day for one hour with 2% milk solution on two consecutive days (days 28 and 29), twice a day for one hour with 2% milk solution on a single day (day 29) or twice a day for 90 min with 1% milk solution on two consecutive days (days 28 and 29). The mice were treated
Chapter 2 Materials and Methods

either with Tiotropium Bromide (0.1 mg/kg, 1 mg/kg or 10 mg/kg in 50 µl or 25 µl 1x PBS) or with 1x PBS (vehicle control) by intranasal application of the compound either once on the day before the aerosol challenge or five times once a day, between day 25 and day 29. The mice were lethally anesthetized on day 31. There were four groups of mice per experiment: naive mice as negative controls, PBS-treated mice as vehicle controls and two groups of mice treated with two different concentrations of Tiotropium Bromide.

Figure 2.1 Experimental setup - First experimental approach for acute asthma.

Figure 2.2 Experimental setup - Second experimental approach for acute asthma
2.1 Experimental design of the in vivo model

**Figure 2.3** Experimental setup - Third experimental approach for acute asthma

**Experimental approach for Tiotropium Bromide (Ba 679 BR) treatment in the memory model of experimental allergic asthma**

In the memory model of disease, acute disease was induced as mentioned above and after a recovery phase of 113 days (~three and a half month), disease relapse was induced by allergen challenge on two consecutive days with 1% milk solution (days 142 and 143) for 90 min. The mice were treated on five consecutive days between the days 139 and 143 by intranasal instillation of the compound, either 1 mg/kg or 0.1 mg/kg, diluted in 25 µl 1xPBS. The vehicle group were administered 25 µl 1x PBS only. Mice were lethally anesthetized 72h after the last treatment. There were four groups of mice per experiment: naive mice as negative controls, PBS-treated mice as vehicle controls and two groups of mice treated with two different concentrations of Tiotropium Bromide.

**Figure 2.4** Experimental setup - Memory model of allergic asthma
Chapter 2 Materials and Methods

Experimental approach for MAM-06.301 treatment in the acute model of experimental allergic asthma

Mice were sensitized and challenged with low-fat powder milk. They were immunized by intraperitoneal injection of 10 µg milk in 200 µl 0.9% NaCl, on days 0 and 21. The mice were exposed to aerosolized milk solution dissolved in 0.9% NaCl, produced by an ultrasonic nebulizer, following two different protocols. Either twice a day for one hour with 2% milk solution on two consecutive days (days 28 and 29) or twice a day for one hour with 2% milk solution on a single day (day 29). The mice were treated by intraperitoneal injection on 5 consecutive days (days 26 to 30), either with MAM-06.301 (1 mg/kg once or twice a day dissolved in 0.9% NaCl), with 200 µl 1x PBS (vehicle control group) or with 1 mg/kg dexamethasone (treatment control group). Mice were lethally anesthetized 24h after the last treatment. There were five groups of mice per experiment: naive mice as negative controls, PBS-treated mice as vehicle controls, dexamethasone-treated mice as treatment controls and two groups of mice treated with MAM-06.301, either once (O.D) or twice (b.i.d) a day.

Figure 2.5 Experimental setup - First experimental approach for acute asthma
2.1 Experimental design of the in vivo model

2.1.3 Setup of experimental allergic asthma, Ba 679 BR treatment protocol and measurement of AHR in the BALB/c mouse strain

Mice were sensitized and challenged with ovalbumin. They were immunized by intraperitoneal injection of 10 µg ovalbumin in 200 µl 1x PBS, on days 0 and 21. The mice were exposed to aerosolized 1% ovalbumin solution dissolved in 1x PBS, produced by an ultrasonic nebulizer. The mice were administered different doses of Ba 679 BR (0.00001, 0.0001, 0.001, 0.01, 0.1, 1 and 10 mg/kg) or 1x PBS intratracheally, once. AHR was measured using a whole body plethysmography at different timepoints after the treatment day (5h, 24h, 48h, 72h, 96h, 7d, 9d and 27d).
14d). The response of the airways to inhaled methacholine at different concentrations ranging from 2.5 to 75 mg/ml was recorded. AHR was expressed as enhanced pause (PenH), a calculated value that correlates with pulmonary resistance.

### 2.2 Processing of bronchoalveolar fluid, blood and lung tissue after the assessment

#### 2.2.1 Serum collection

Blood was obtained by heart puncture and clotted at room temperature. After centrifugation of the clotted blood (15min, 5000 rpm), the sera were separated, kept in Eppendorf’s tubes and stored at -20°C until use.

#### 2.2.2 Collection of bronchoalveolar lavage fluid (BALF)

To collect the BALF, the trachea was cannulated using catheter and syringe and the lungs were lavaged with 2 - 2.5 ml of 1x PBS. The BALF were then centrifuged at room temperature (10 min, 1500 rpm). Approximately 1.5 ml of supernatant were gained, transferred into a new Eppendorf tube and kept at -20°C until use (see 2.5). The cell pellets were resuspended in 0.5 ml of 1x PBS by vortexing the samples. A small fraction of the BALF was mixed with trypan blue dye for viable cell counting on a Neubauer haemocytometer. The other fraction of the BAL fluid was used for differential cell count.

#### 2.2.3 Preparation of BALF slides by cytopsin centrifugation

The fractions of BALF kept for differential cell count were processed centrifuged again at room temperature (10 min, 1500 rpm), the supernatant thrown away and the cell pellets resuspended in 1x PBS. The resuspension volume is calculated for each sample, so that 100 µl of the cell suspension contains 1.10^5 cells. The slides were fixed on a metal holder with a plastic funnel, 100 µl of each sample pipetted into the corresponding funnel and centrifuged using a Cytospin3 (Shandon).
2.3 Histopathological staining methods

2.2.4 Analysis of leukocyte subsets by differential cell count of BALF monolayer after Wright-Giemsa staining

The BALF cytospins were first incubated for 5 min in methanol to fix the tissue, then in May - Grünwald solution for 15 min. After a washing step in aqua destillata (aqua dest), the slides were incubated for 20 min in Giemsa solution, washed again in aqua dest and air dried. The differential cell counts were determined based on morphological criteria of the different cell types, namely macrophages, eosinophils, neutrophils and lymphocytes. The counts were performed under light microscope at 100x magnification and a total of 400 cells were counted.

2.2.5 Fixation of the lungs and embedding in paraffin

Lungs with trachea were removed and fixed for at least 4 hours in 4% paraformaldehyde. The tissue was dehydrated in an automated process and embedded in paraffin.

2.3 Histopathological staining methods

2.3.1 Deparaffinazation, dehydration and rehydration of the lung tissue

For all histopathological stainings, 3 to 4 µm-thick lung sections were cut from paraffin blocks with a microtom and fixed on slides. The deparaffinazation step of the lung sections consisted in two steps. Firstly, the incubation in xylol, which plays the role of intermedium, for 20 min, secondly, the incubation at 60-65°C for 30 min in an oven. After deparaffinazation, the sections were dehydrated using the following alternative ascendant ethanol gradient: either 1 min in 100%, 1 min in 96% and 1 min in 80% ethanol or 1 min in 100%, 1 min in 70% and 1 min in 50% ethanol. Both gradients worked in an equivalent manner. The last step of all stainings was the rehydration step and consisted in a 5 min incubation of the slides in a descending ethanol gradient: 50/ 80%, 70/ 96% and 100% ethanol. The slides were then washed intensively in aqua dest, until the level of water could slide horizontally from the slides.
2.3.2 Periodic acid - Schiff staining of lung sections for mucus producing goblet cells

After deparaffinazation, dehydration and washing of the lung sections, the latter were incubated for 10 min in 0.5% periodic acid and washed in aqua dest 5 to 6 times. The slides were then incubated in the Schiff’s reagent, which was previously put in the fridge (4°C), for 30 min at 60°C in the oven. Follow then 6 min incubation in a fresh prepared SO$_2$ water and a step, were the slides were washed for 2-3 min under luke warm running water. The sections are finally incubated for 19 sec in the papanicouleous solution and washed directly after this, abundantly under running luke warm water until the water in the reservoir becomes clear. The lung sections were rehydrated and incubated for 5 min in n-butylacetate. The slides were then kept for drying at air temperature for 5 min, mounted with a synthetic resin solution (Pertex) and covered with appropriate cover slips. The tissues were analyzed at a 20x magnification. To quantify the degree of mucus production, 10 representative fields of the central airway region of each lung section were chosen and graded from 0 to 5. The grade 0 was given when an airway was completely free from mucus, 5 when all goblet cells of an airway produced mucus massively. If a field contained more than one airway, the mean of the individual airway grade was calculated. The grade of mucus production of each mouse lung consisted therefore in the mean of 10 fields in a lung section.

In one experiment histological analyses were quantified differently, detailed information are given in 3.3.1.

2.3.3 LUNA staining of lung sections for the presence of eosinophils in infiltrates

The paraffin lung sections were deparaffinized, dehydrated and washed. These were then placed for 7.5 min in the working solution. To prepare the working solution, 200 ml of a 1% haematoxylin solution, 8 ml of a 30% ferric chloride solution, 200 ml, 1% acid water and 45 ml of a 1% Biebrich Scarlet solution (Ponceau S) were mixed together and filtered in a bottle. The next step consisted in dipping 3-4 times the sections in a 1% acid alcohol solution, followed by an intense washing under running tap water. The slides were then dipped into a 0.5% lithium carbonate solution until the colour of the sections changed from pinkish to bluish (3-5 dips) and washed.
2.4 Detection of milk-specific IgG1 in the serum by ELISA

abundantly under tap water. The lung sections were rehydrated, dried at RT for 5 min, mounted with Pertex and covered with cover slips. The tissues were analyzed at 40x magnification. The degree of eosinophil infiltration around airways, blood vessels and parenchyma was given by the mean eosinophil number of 10 peripheral foci of a lung section.

In one experiment histological analyses were quantified differently, detailed information are given in 3.3.1.

2.3.4 Haematoxylin and eosin staining of lung section for cell infiltration

The paraffin lung sections were deparaffinized, dehydrated and washed. The slides were incubated for 5 min in haematoxylin solution, washed by dipping ca. 10 times in tap water, incubated for 5 sec in 1% acid chloride water and washed again for 5 min under tap water followed by 5 min in aqua dest. Place then the slides in 50% ethanol for 30 sec, in 70% ethanol for 30 sec and in eosin for 30 sec. To remove the excess of eosin, rinse the slides by dipping in tap water. The lung sections were rehydrated, dried at RT for 5 min, mounted with Pertex and covered with cover slips. Pictures showing infiltration of the lung were made at 4x, 20x and 40x magnification.

In one experiment histological analyses were quantified differently, detailed information are given in 3.3.1.

2.4 Detection of milk-specific IgG1 in the serum by ELISA

Each assay was performed on 3 days. Between each step, the wells were washed by pipetting three times with an ELISA washing buffer containing per liter aqua dest, 6.06 g Tris and 2 ml Tween20. The sera (first Ab), the second Ab and the streptavidin-horseradish peroxidase (SA-HRP) were diluted in 0.5% Tween20 solution in 1x PBS. 96 well plates with flat bottoms were coated with 10 μg/ml milk solution in 1x PBS overnight at 4°C. On day 2, unspecific binding sites were blocked by 0.5% Tween20 for 2h at room temperature followed by the addition of the sera. Each serum was added in duplicate in the first row of each plate at a starting dilution of 1:100 or
Chapter 2 Materials and Methods

1:50. A serial dilution of 1:2 was performed. Each plate included a serum-free blank as negative and a known positive serum for specific IgG1 as a positive control. The sera were incubated overnight at 4°C. On day 3, the second Ab (IgG1 biotinylated) was incubated for 2h at 4°C at a dilution of 1:5000. Then, the streptavidin-bound enzyme was added at a dilution of 1:8000 and incubated for 1h at room temperature. To detect the milk-specific Abs, the substrate was added. TMB was mixed at equal volumes and incubated for 10 min in the dark. Finally, the plates were analyzed on an ELISA reader (Spectra Max M5) by the Soft Max Pro 8 software.

2.5 Detection of inflammatory cytokines in BALF supernatant by ELISA

These ELISA assays were performed by Marinomed. Eotaxin, TNF-α, IL-4, IL-5 and IL-13 concentrations were determined in undiluted BALF supernatants kept after BALF collection (details see 2.2.2).

2.6 Statistical analysis

Statistical analyses were performed using the GraphPad Prism 5.0 statistical software for windows (GraphPad Prism, San Diego, CA). Values of absolute cell number and percentage of BAL cells are expressed as means ± SEM. Score values of histological gradings are expressed as medians. Data expressed as means ± SEM were evaluated by two-tailed unpaired T-test and data expressed as medians were analyzed using the non-parametric Mann-Whitney test. A P value of <0.05 was considered significant.
Chapter 3

Results

3.1 Effect of Tiotropium Bromide on an acute model of allergic asthma

We used different approaches to test Tiotropium Bromide by varying the aerosol challenges, the dose and the frequency of administration of the drug.

3.1.1 Effect of Tiotropium Bromide treatment on AHR induced in an acute model of allergic asthma

AHR to methacholine was assessed by a working group from a laboratory possessing the adequate apparatus. As the C57BL/6 mouse model of allergic asthma has attenuated AHR\(^\text{4,56}\), this parameter was measured on the classical ovalbumin-induced acute asthma model in BALB/c mice (for details see 2.1.3).

The mice inhaled increasing concentration of methacholine at different timepoints after instillation of Tiotropium. The control group was administered PBS and developed strong AHR. Interestingly, Tiotropium seems to have a highly preventive effect on AHR development in a dose-dependent manner. In the concentration range from 0.001 to 10 mg/kg but not below, Tiotropium administration protects mice from developing AHR in response to methacholine. The time period in which the mice are completely protected from AHR is dose-dependent as well. In fact, drug concentrations between 0.01 and 10 mg/kg prevented from AHR within 7 days following drug administration, whereas concentrations between 0.001 and 0.01
mg/kg gave complete protection only up to 72h after Tiotropium treatment. AHR is re-established 14 days after drug administration in the treated mice, when potential to develop AHR was already decreased in the positive controls. Tiotropium has an impressive protective effect on AHR development in the mouse model used, and exhibits a dose- and time- dependent effect.

3.1.2 First experimental approach of the acute model of experimental asthma

In the first experimental approach, the mice were given one dose of either 1 or 10 mg/kg Tiotropium Bromide intranasally, one day before the first allergen challenge and were aerosolized with a 2% milk solution twice a day on two consecutive days. The mice were sacrificed four days after the treatment day (see Fig.2.1).

Effect of Tiotropium Bromide treatment on inflammatory cells in BALF

The BALF of naive mice is characterized by a high percentage of macrophages (> 95%) and a low percentage of eosinophils (< 1 %), neutrophils (< 1 %) and lymphocytes (< 3%) (see Fig.3.1a). Furthermore, naive mice have a highly significant lower number of total cells in their BALF (< 500,000 cells) compared to the asthmatic mice (between 2.5 and 3.10^6 cells, see Fig.3.1b). This disease protocol induced a very important recruitment of eosinophils into the airways, as they represent 90% of the cells in the BALF of asthmatic mice. The difference in the cellular BALF constitution is significant between asthmatic and naive mice for macrophages, neutrophils and lymphocytes, and highly significant for eosinophils. The treatment administered did not decrease the percentage of eosinophils in BALF and did not have significant effects on the total cell number in the BALF or on the number of eosinophils or lymphocytes. Interestingly, the 1 mg/kg but not the 10 mg/kg treatment had an effect on the number of neutrophils.

Effect of Tiotropium Bromide treatment on inflammation in lung tissue

Lungs of naive mice are healthy. Severe pulmonary inflammation with eosinophilic infiltration occurs in the lungs of animals with acute asthma as revealed by histological
3.1 Effect of Tiotropium Bromide on an acute model of allergic asthma

These lungs exhibit very significant goblet cell hyperplasia and mucus production in the airways compared with naive lungs (see Fig.3.2a and Fig.3.4 A and B). There is an important cell infiltration forming inflammatory foci around blood vessels and airways (see Fig.3.6 B). More specifically, we can confirm an infiltration of mostly eosinophilic nature spreading from the foci into the parenchyma (see Fig.3.2b and Fig.3.5B). Surprisingly, the treatment did not have beneficial effects on any aspect of the inflammation as determined by quantitative grading scores (see Fig.3.4 C and D and Fig.3.5 C and D) and qualitative observation of lung infiltration (see Fig.3.6 C and D).

**Effect of Tiotropium Bromide treatment on allergen-specific IgG1 antibodies**

In the sera of naive mice, no milk-specific IgG1 antibodies are detected. The IgG1 level in asthmatic mice is low (see Fig.3.3) but confirms the results of previous experiments performed in our laboratory on the same model. In fact, allergen-specific IgG1 serum level in asthmatic mice is higher in ovalbumin-sensitized BALB/c mice than in milk-sensitized C57BL/6 mice. Nevertheless, a significant difference between the IgG1 level of naive and asthmatic mice is detected at the highest serum dilution (1:100). However, none of the Tiotropium doses had an effect on the allergen-specific antibody level. In the 10 mg/kg-treated group more than half of the mice developed higher antibody levels than the asthmatic mice, but this effect turns out not to be significant. The groups are heterogeneous concerning their IgG1 production.
Chapter 3 Results

(a) Percentage of individual leukocyte populations in BAL

(b) Total cell counts in BAL

Figure 3.1 Effect of Tiotropium on inflammatory cells in BAL - First experimental approach for acute asthma. Data are expressed as means ± SEM and represent the values of two experiments performed under the same conditions. Values from naive mice represent pooled values of all experiments done with Tiotropium Bromide. Differences between groups respectively to the cell types were calculated with a two-tailed unpaired t-test. For total cell number, naive vs vehicle p<0.0001; for macrophage numbers, naive vs vehicle p=0.0495; for eosinophil numbers, naive vs vehicle p<0.0001; for neutrophil numbers, naive vs vehicle p=0.0130, vehicle vs Rx 1 mg/kg p=0.0093; for lymphocyte numbers, naive vs vehicle p=0.0342.
3.1 Effect of Tiotropium Bromide on an acute model of allergic asthma

(a) Mucus production in the airway

(b) Eosinophilic infiltration in the peripheral airways

Figure 3.2 Effect of Tiotropium on mucus production and eosinophil number - First experimental approach for acute asthma. Data are expressed as medians and represent the values of two experiments performed under the same conditions. Values from naive mice represent pooled values of all experiments done with Tiotropium Bromide. Differences between groups were calculated with a Mann-Whitney test. For PAS, naive vs vehicle p=0.0039; for LUNA, naive vs vehicle p=0.0003.
Figure 3.3 Milk-specific IgG1 antibody detection by ELISA - First experimental approach for acute asthma. Data are expressed as means ± SEM and represent the values of two experiments performed under the same conditions. Values from naive mice represent pooled values of all experiments done with Tiotropium Bromide. Differences between groups were calculated for each serum dilution with a two-tailed unpaired t-test. At 1:100, naive vs vehicle p=0.0211.
3.1 Effect of Tiotropium Bromide on an acute model of allergic asthma

**Figure 3.4** Lung histology in naive (A), asthmatic (B) and Tiotropium-treated (C and D) mice. Representative photomicrographs of PAS whole-lung sections for goblet cells of the airway epithelium - First experimental approach for acute asthma. Objective lens x200

**Figure 3.5** Lung histology in naive (A), asthmatic (B) and Tiotropium-treated (C and D) mice. Representative photomicrographs of LUNA whole-lung sections for eosinophil staining - First experimental approach for acute asthma. Objective lens x600
Figure 3.6 Lung histology in naive (A), asthmatic (B) and Tiotropium-treated (C and D) mice. Representative photomicrographs of H&E whole-lung sections for cell infiltration - First experimental approach for acute asthma. Objective lens x40, x200 and x400
3.1.3 Second experimental approach of the acute model of experimental asthma

In the second set of experiment, the mice were given five doses of either 1 or 0.1 mg/kg Tiotropium Bromide intranasally and were aerosolized with 2% milk solution twice a day on a single day. Inflammation status was assessed 48h after the last treatment and the aerosol challenge. After the results of the first set of experiments, we speculated that an eventual beneficial effect of the drug might not be visible due to the severity of the disease induced (90% eosinophils in the BALF). We decided therefore to change the aerosol challenge protocol in order to reach a lower degree of inflammation and the treatment dose by increasing the frequency of drug administration. We sacrificed the mice 48h instead of 4 days after the last treatment, as we supposed that this lapse of time was more adequate to detect the drug effect (see Fig.2.2).

Effect of Tiotropium Bromide treatment on inflammatory cells in BALF

The effects of the change in the aerosol protocol on the degree of inflammation were disappointing and below our expectations. Only half of the mice among the asthmatic group developed sufficient degree of inflammation in the airways as revealed by raw data on the cellular BALF constitution. Therefore, the asthmatic control group is characterized by a high heterogeneity between the mice. The responding mice developed as expected a lower percentage of eosinophils (~40% of BALF cells) than the mice in the first experimental approach described before (see Fig. 3.1a). In all groups, the fraction of neutrophils and lymphocytes is low and stays under 2% (see Fig. 3.7a). Despite the high variability in the asthmatic group, the number of eosinophils in the BALF differs very significantly from the naive group but does not exceed 500,000 cells (see Fig. 3.7b). With this disease protocol, no difference in the total BALF cell number as well as in the number of macrophages, neutrophils and lymphocytes is observed between the two control groups. A high heterogeneity is observed in both treated groups as well. Their mean eosinophil percentage varies around 30% compared to a mean percentage of 20% in the asthmatic group. The treatments had no effect on any cell type number in the BALF compared to the asthmatic group.
Chapter 3 Results

Effect of Tiotropium Bromide treatment on inflammation in lung tissue

The histological analysis confirmed the BALF results. The non-responder mice from the asthmatic group, with almost no eosinophils in their BALF, have a relatively low pulmonary inflammation and eosinophilic cell infiltration compared to the responding half of the group. In the treated groups however, no correlation between eosinophil presence in the BALF and degree of tissue inflammation can be made. In fact, mice with low mucus production and/or low eosinophilic infiltration are not necessarily the mice with low eosinophil percentage in their BALF, which is surprising. There is no significant difference in the degree of mucus production between naive and asthmatic mice (see Fig.3.8a and Fig.3.10 A and B) indicating that this disease protocol is not well adapted for this aspect of disease inflammation. The eosinophilic infiltration in the lung periphery differs very significantly between the control groups (see Fig.3.8b and Fig. 3.11 A and B) but the treatments had no effect on the eosinophil number when compared to the asthmatic group (see Fig. 3.11 C and D). The qualitative analysis of lung infiltration confirms the quantitative results, as administration of the drug did not seem to have any effect on the grade of cell infiltration in the lung (see Fig.3.12).

Effect of Tiotropium Bromide treatment on allergen-specific IgG1 antibodies

The general antibody levels are lower than in the first approach, which confirms the attenuated effect of the modified aerosol challenge. The optical density does not exceed the value of 1, even for the asthmatic mice (see Fig.3.9). However, at a 1:100 and 1:200 serum dilution, the IgG1 level of asthmatic mice differs significantly from the level in naive mice. The non-responder animals from the asthmatic group did not develop specific antibodies like expected. The Tiotropium treatment did not have any significant influence on the allergen-specific antibody level in the serum of the treated mice. The treated groups are heterogeneous concerning their antibody response and in each treated group respectively, one mouse developed higher IgG1 levels than the asthmatic mice, which is disappointing. Nevertheless, the high variability among the groups confirms the tendency observed in the histological and cellular analysis of lungs and airways and could account for a failed detection of treatment effects, if there exists any.
3.1 Effect of Tiotropium Bromide on an acute model of allergic asthma

**Figure 3.7** Effect of Tiotropium on inflammatory cells in BAL - Second experimental approach for acute asthma. Data are expressed as means ± SEM and represent the values of two experiments performed under the same conditions. Values from naive mice represent pooled values of all experiments done with Tiotropium Bromide. Differences between groups respectively to the cell types were calculated with a two-tailed unpaired t-test. For eosinophil numbers, naive vs vehicle p=0.0053.

(a) Percentage of individual leukocyte populations in BAL

(b) Total cell counts in BAL
Chapter 3 Results

(a) Mucus production in the airway

(b) Eosinophilic infiltration in the peripheral airways

Figure 3.8 Effect of Tiotropium on mucus production and eosinophil number - Second experimental approach for acute asthma. Data are expressed as medians and represent the values of two experiments performed under the same conditions. Values from naïve mice represent pooled values of all experiments done with Tiotropium Bromide. Differences between groups were calculated with a Mann-Whitney test. For LUNA, naïve vs vehicle p=0.0013.
3.1 Effect of Tiotropium Bromide on an acute model of allergic asthma

Figure 3.9 Milk-specific IgG1 antibody level detected by ELISA - Second experimental approach for acute asthma. Data are expressed as means ± SEM and represent the values of two experiments performed under the same conditions. Values from naive mice represent pooled values of all experiments done with Tiotropium Bromide. Differences between groups were calculated for each serum dilution with a two-tailed unpaired t-test. At 1:100, naive vs vehicle p=0.0154; at 1:200, naive vs vehicle p=0.0451.
Chapter 3 Results

Figure 3.10 Lung histology in naive (A), asthmatic (B) and Tiotropium-treated (C and D) mice. Representative photomicrographs of PAS whole-lung sections for goblet cells of the airway epithelium - Second experimental approach for acute asthma. Objective lens x200

Figure 3.11 Lung histology in naive (A), asthmatic (B) and Tiotropium-treated (C and D) mice. Representative photomicrographs of LUNA whole-lung sections for eosinophil staining - Second experimental approach for acute asthma. Objective lens x400
3.1 Effect of Tiotropium Bromide on an acute model of allergic asthma

Figure 3.12 Lung histology in naïve (A), asthmatic (B) and Tiotropium-treated (C and D) mice. Representative photomicrographs of H&E whole-lung sections for cell infiltration - Second experimental approach for acute asthma. Objective lens x40, x200 and x400
Chapter 3 Results

3.1.4 Third experimental approach of the acute model of experimental asthma

As the protocol of the second experimental approach turned out to be an unadapted model for our purpose, we modified the aerosol challenge protocol and challenged the mice with 1% milk solution twice a day on two consecutive days, in order to establish a more stable disease in the mice. The treatment protocol was kept identical and the mice were sacrificed 72h after the last challenge and the last treatment respectively (see Fig.2.3).

Effect of Tiotropium Bromide treatment on inflammatory cells in BALF

The majority of the mice from the asthmatic control group responded to the disease induction protocol with approximately 70% eosinophils in the BALF. One mouse distinguishes itself from the other animals of the group with 15% eosinophils in its BALF and contributes to distort the mean percentage down to 40% (see Fig.3.13a). In the group treated with 1 mg/kg, one mouse is out of range compared with the animals of the group and shows a low eosinophil percentage (6%). I hypothesize this effect to be random, as we know that some mice do not respond to the disease protocol, more than being a real significant effect of the drug treatment. In the group treated with 0.1 mg/kg, the drug seems to decrease the percentage of eosinophils in BALF, from 70% (if we consider only the fraction of the mice in the asthmatic group which responded) to 40%. Nevertheless, this decrease would have to be confirmed by further experiments, as a random effect cannot be put by side. In the group treated with 1 mg/kg, half of the mice have an increased neutrophil percentage (up to 20%). Beside this, the fraction of neutrophils and lymphocytes is low and stays under 2%. Although in the percental description of the cell distribution only one animal has a noticeable low eosinophil ratio, the total cell number exhibits a very big variation between the mice. The non-responder mouse has the lowest number of total cells (240,000 cells) but the rest varies between 480,000 and $8.10^6$ cells (see Fig.3.13b). Nevertheless, the total number of BALF cells, the number of eosinophils and of lymphocytes of the asthmatic mice differs significantly from the cell number in the BALF of naive mice. The treatment administered did not influence the cellular BALF composition of the mice compared to the asthmatic mice. Notice that there is a high variability among the mice treated with 1 mg/kg.
3.1 Effect of Tiotropium Bromide on an acute model of allergic asthma

Effect of Tiotropium Bromide treatment on inflammation in lung tissue

The mucus production in the lung is very significantly (see Fig.3.14a and Fig.3.16 A and B) and the eosinophilic infiltration significantly different (see Fig.3.14b and Fig.3.17 A and B) between the two control groups. These parameters were not attenuated by the treatment, independently of the dose given (Fig.3.16 and Fig.3.17 C and D). Cell infiltration grade assessed by H&E staining confirmed the quantitative observations (see Fig.3.18).

Effect of Tiotropium Bromide treatment on allergen-specific IgG1 antibodies

Although the intensity of inflammation was expected to increase compared to the previous setup, the general level of IgG1 is suspiciously very low and the IgG1 level in asthmatic mice does not differ significantly from the level in the naive mice (see Fig.3.15). Though, in the sera from two of the three responder mice from the asthmatic group, absolutely no IgG1 is detected.
Chapter 3 Results

(a) Percentage of individual leukocyte populations in BAL

(b) Total cell counts in BAL

Figure 3.13 Effect of Tiotropium on inflammatory cells in BAL - Third experimental approach for acute asthma. Data are expressed as means ± SEM and represent the values of two experiments performed under the same conditions. Values from naive mice represent pooled values of all experiments done with Tiotropium Bromide. Differences between groups respectively to the cell types were calculated with a two-tailed unpaired t-test. For total cell number, naive vs vehicle p=0.0245; for eosinophil numbers, naive vs vehicle p=0.0456; for lymphocytes, naive vs vehicle p=0.0456.
3.1 Effect of Tiotropium Bromide on an acute model of allergic asthma

(a) Mucus production in the airway

(b) Eosinophilic infiltration in the peripheral airways

Figure 3.14 Effect of Tiotropium on mucus production and eosinophil number - Third experimental approach for acute asthma. Data are expressed as medians and represent the values of two experiments performed under the same conditions. Values from naive mice represent pooled values of all experiments done with Tiotropium Bromide. Differences between groups were calculated with a Mann-Whitney test. For PAS, naive vs vehicle p=0.0032; for LUNA, naive vs vehicle p=0.0131.
Figure 3.15 Milk-specific IgG1 antibody detection by ELISA - Third experimental approach for acute asthma. Data are expressed as means ± SEM and represent the values of two experiments performed under the same conditions. Values from naive mice represent pooled values of all experiments done with Tiotropium Bromide. Differences between groups were calculated for each serum dilution with a two-tailed unpaired t-test.
3.1 Effect of Tiotropium Bromide on an acute model of allergic asthma

Figure 3.16 Lung histology in naive (A), asthmatic (B) and Tiotropium-treated (C and D) mice. Representative photomicrographs of PAS whole-lung sections for goblet cells of the airway epithelium - Third experimental approach for acute asthma. Objective lens x200

Figure 3.17 Lung histology in naive (A), asthmatic (B) and Tiotropium-treated (C and D) mice. Representative photomicrographs of LUNA whole-lung sections for eosinophil staining - Third experimental approach for acute asthma. Objective lens x400
Figure 3.18 Lung histology in naive (A), asthmatic (B) and Tiotropium-treated (C and D) mice. Representative photomicrographs of H&E whole-lung sections for cell infiltration - Third experimental approach for acute asthma. Objective lens x40, x200 and x400
3.2 Effect of Tiotropium Bromide on a memory model of allergic asthma

We investigated the effect of the drug administration during another phase of disease simulation. In this memory model, an acute asthma phase was induced like described before. For acute disease, the mice were aerosolized with a 2% milk solution twice a day on a single day. After a rest of approximately 110 days (~3.5 months) that simulates disease remission, the mice were rechallenged with 1% milk solution twice a day on two consecutive days for disease exacerbation. We treated the mice intranasally with 1 mg/kg or 0.1 mg/kg of the drug on five consecutive days, before disease relapse. The mice were sacrificed 72h after the last challenge and the last treatment respectively (see Fig.2.4).

3.2.1 Effect of Tiotropium Bromide treatment on inflammatory cells in BALF

All the so-called “memory mice”, meaning the mice with a relapsing disease, present around 75% eosinophils in their BALF (see Fig.3.19a). In each of both treated groups, the percentage of eosinophils is constant, the groups are homogeneous respectively. The mean percentage of eosinophils of the animals treated with 1 mg/kg slightly decreases to 60%. No effect on the eosinophil fraction is detected in the group receiving 0.1 mg/kg, compared to the asthmatic group. Neutrophils and lymphocytes do not exceed 3% of the total cells. The total cell number in the BALF of naive mice (around 500,000 cells) differs highly significantly from the extremely high number of cells counted in the BALF of asthmatic mice, which varies between 5 and 10.10^6 (see Fig.3.19b). The number of neutrophils, macrophages, and eosinophils, but not lymphocytes, differs significantly to highly significantly respectively between asthmatic and naive mice. Interestingly, the 1 mg/kg treatment have a significant effect on the total BALF cell number and on the number of eosinophils compared to the positive control group.


Chapter 3 Results

3.2.2 Effect of Tiotropium Bromide treatment on inflammation in lung tissue

The mucus production in the lung differs very significantly between the two control groups (see Fig.3.20a and Fig.3.22 A and B) as well as the eosinophilic infiltration (see Fig.3.20b and Fig.3.23 A and B). These parameters were not attenuated by the treatment, independently of the dose given (Fig.3.22 and Fig.3.23 C and D). An effect of the 1 mg/kg treatment on the eosinophil number in the lung periphery is not big enough to be significantly different from the number in the lungs of asthmatic mice, which is confirmed by the qualitative observations of the H&E staining (see Fig.3.24).

3.2.3 Effect of Tiotropium Bromide treatment on allergen-specific IgG1 antibodies

The analysis of the asthmatic mice sera indicate, as in my previous experiments, a very low level of IgG1. Except for one mouse of the group treated with 1 mg/kg of the compound, the IgG1 level in the sera of the treated animals is elevated compared to the IgG1 level in the sera of asthmatic mice (see Fig.3.21). There is a significant difference in the IgG1 levels between asthmatic and naive mice at a 1:100 dilution but not at 1:50, probably because of the high variability among the group at a 1:50 dilution. At 1:50 and 1:100, the IgG1 levels of the 0.1 mg/kg treated mice differ significantly from the levels in the asthmatic group.
3.2 Effect of Tiotropium Bromide on a memory model of allergic asthma

(a) Percentage of individual leukocyte populations in BAL

(b) Total cell counts in BAL

Figure 3.19 Effect of Tiotropium on inflammatory cells in BAL - Memory model of experimental asthma. Data are expressed as means ± SEM and represent the values of two experiments performed under the same conditions. Values from naive mice represent pooled values of all experiments done with Tiotropium Bromide. Differences between groups respectively to the cell types were calculated with a two-tailed unpaired t-test. For total cell number, naive vs vehicle p<0.0001, vehicle vs Rx 1 mg/kg p=0.0175; for macrophage numbers naive vs vehicle p=0.0021, for eosinophil numbers, naive vs vehicle p<0.0001, vehicle vs Rx 1 mg/kg p=0.0033; for neutrophils, naive vs vehicle p=0.0119.
Chapter 3 Results

(a) Mucus production in the airway

(b) Eosinophilic infiltration in the peripheral airways

Figure 3.20 Effect of Tiotropium on mucus production and eosinophil number - Memory model of experimental asthma. Data are expressed as medians and represent the values of two experiments performed under the same conditions. Values from naive mice represent pooled values of all experiments done with Tiotropium Bromide. Differences between groups were calculated with a Mann-Whitney test. For PAS, naive vs vehicle p=0.0032; for LUNA, naive vs vehicle p=0.0013.
3.2 Effect of Tiotropium Bromide on a memory model of allergic asthma

Figure 3.21 Milk-specific IgG1 antibody detection by ELISA - Memory model of allergic asthma. Data are expressed as means ± SEM and represent the values of two experiments performed under the same conditions. Values from naïve mice represent pooled values of all experiments done with Tiotropium Bromide. Differences between groups were calculated for each serum dilution with a two-tailed unpaired t-test. At 1:50, vehicle vs Rx 0.1 mg/kg p=0.0356; at 1:100, naïve vs vehicle p=0.0484, vehicle vs Rx 0.1 mg/kg p=0.0398.
Chapter 3 Results

**Figure 3.22** Lung histology in naive (A), asthmatic (B) and Tiotropium-treated (C and D) mice. Representative photomicrographs of PAS whole-lung sections for goblet cells of the airway epithelium - Memory model of allergic asthma. Objective lens x200

**Figure 3.23** Lung histology in naive (A), asthmatic (B) and Tiotropium-treated (C and D) mice. Representative photomicrographs of LUNA whole-lung sections for eosinophil staining - Memory model of allergic asthma. Objective lens x400
3.2 Effect of Tiotropium Bromide on a memory model of allergic asthma

Figure 3.24 Lung histology in naïve (A), asthmatic (B) and Tiotropium-treated (C and D) mice. Representative photomicrographs of H&E whole-lung sections for cell infiltration - Memory model of allergic asthma. Objective lens x40, x200 and x400
Chapter 3 Results

3.3 Effect of MAM-06.301 on an acute model of allergic asthma

Three experimental approaches were used to test this compound. The protocols differ in the aerosol challenges and in the frequency of the treatments but neither in the dose of MAM-06.301 nor in the dose of dexamethasone administered. Both were always used at a concentration of 1 mg/kg.

3.3.1 First experimental approach of the acute model of experimental asthma

In the first set of experiment the mice were given MAM-06.301 intraperitoneally on five consecutive days and aerosolized with 2% milk solution twice a day on two consecutive days. The mice were assessed 72h after the last challenge and 24h after the last treatment (see Fig.2.5). Like mentioned in 2.3, this experiment was carried out by my colleague Lamia El-Housseiny, whose quantitative histological analysis criteria’s are different than mine. Detailed information is given below.

Effect of MAM-06.301 treatment on inflammatory cells in BALF

In this experiment, the control groups differ significantly from each other concerning the eosinophil number in the BALF (see Fig.3.25b). Whereas these cells are almost absent in naive mice, asthmatic mice have over $2.10^6$ eosinophils in their BALF. Compared to the vehicle group, dexamethasone treatment reduced very significantly the number of eosinophils down to 100,000. The trend between control groups is reflected also in terms of percentage. In fact, asthmatic mice have around 70% and dexamethasone-treated mice around 20% eosinophils in their BALF (see Fig.3.25a). In none of the groups, neutrophils are present and lymphocyte number does not differ between naive, vehicle and dexamethasone-treated groups. However, although MAM-06.301 treatment affected neither the eosinophil number nor its percentage in BALF, the number of lymphocytes differs significantly from the BALF of asthmatic mice in the MAM-06.301-treated group.
3.3 Effect of MAM-06.301 on an acute model of allergic asthma

Effect of MAM-06.301 treatment on inflammation in lung tissue

For quantification of eosinophilic infiltration based on LUNA staining, the cells were counted in the parenchyma by distinguishing between central (large airways) and peripheral (small airways) lung first. Further analysis discerned between eosinophils localized around the airway (peribronchial) or around the blood vessel (perivascular). Concerning the eosinophil number, very significant differences between asthmatic and naive mice, and significant differences between asthmatic and dexamethasone-treated mice are observed in both central and peripheral sections of the parenchyma (see Fig.3.26b). Furthermore, these differences are also displayed in areas around airways in blood vessels. Interestingly, MAM-06.301 affects significantly the number of eosinophils in central and peripheral lung, as well as around the airways. However, it has no effect on eosinophils around the blood vessels.

The intensity of mucus production in the airways was quantified by determining the amount of positive stained goblet cells (which are the cells producing mucus) in the totality of the lung section and furthermore by differentiating between mucus production in central and peripheral airways. With this disease model, the mucus production differs very significantly between asthmatic and naive mice from the central to the peripheral located airways (see Fig.3.26a). MAM-06.301 has a significant effect only on the mucus production of small airways situated in the lung periphery.

Using the same criteria for discernment of lung areas, cell infiltration reflected by H&E staining was quantified using the grading method described before. Cell infiltration is very significantly different between asthmatic and naive mice as well as between asthmatic and dexamethasone-treated mice in lung parenchyma (see Fig.3.26c). MAM-06.301 had a very significant effect on the peribronchial infiltration compared to the peribronchial infiltration in the lungs of asthmatic mice.

Effect of MAM-06.301 treatment on inflammatory cytokines in the BALF supernatant

The variability among each group is high concerning all cytokines of interest, namely eotaxin, TNF-α, IL-4, IL-5 and IL-13 (see Fig.3.27). The asthmatic mice seem to have elevated IL-4, IL-5 and IL-13 levels in their BALF supernatant compared
Chapter 3 Results

to naive mice. Surprisingly dexamethasone does not seem to reduce any of these inflammatory mediators. Nevertheless, these analyses indicate an eventual effect of MAM-06.301 on IL-5, which plays a critical role in differentiation of eosinophils and in the sustainability of eosinophilic inflammation.
3.3 Effect of MAM-06.301 on an acute model of allergic asthma

(a) Percentage of individual leukocyte populations in BAL

(b) Total cell counts in BAL

Figure 3.25 Effect of MAM-06.301 on inflammatory cells in BAL - First experimental approach for acute asthma. Data are expressed as means ± SEM. Differences between groups respectively to the cell types were calculated with a two-tailed unpaired t-test. For eosinophil numbers, naive vs vehicle p=0.0008, vehicle vs dex. p=0.0010; for lymphocytes, vehicle vs Rx O.D p=0.0410.
Chapter 3 Results

(a) Mucus production in the airway

(b) Eosinophilic infiltration in the peripheral airways

(c) Cell infiltration in the airways

Figure 3.26 Effect of MAM-06.301 on mucus production and cell infiltration - First experimental approach for acute asthma. Data are expressed as medians. Differences between groups were calculated with a Mann-Whitney test. For PAS, LUNA and H&E in all areas of the lung, naive vs vehicle p=0.0097. For PAS goblet, vehicle vs dex. p=0.0117; for PAS peripheral AW, vehicle vs Rx O.D p=0.0236. For LUNA central lung, lung vehicle vs dex. and vehicle vs Rx O.D p=0.0117; for LUNA peripheral lung, vehicle vs dex. p=0.0119, vehicle vs Rx O.D p=0.0273; for LUNA peribronchial, vehicle vs dex. p=0.0079, vehicle vs Rx O.D p=0.0317; for LUNA perivascular, vehicle vs dex. p=0.0212. For H&E lung and peribronchial, vehicle vs dex. and vehicle vs Rx O.D p=0.0097; for H&E perivascular, vehicle vs dex. p=0.0129.
3.3 Effect of MAM-06.301 on an acute model of allergic asthma

Figure 3.27 Cytokines in BAL supernatant - First experimental approach for acute asthma
3.3.2 Second experimental approach of the acute model of experimental asthma

In the second set of experiment the mice were administered MAM-06.301 intraperitoneally on seven consecutive days, either once (Rx O.D) or twice daily (Rx b.i.d), and aerosolized with 2% milk solution twice a day on two consecutive days. The mice were sacrificed 72h after the last challenge and 24h after the last treatment (see Fig.2.6).

Effect of MAM-06.301 treatment on inflammatory cells in BALF

In the BALF of the asthmatic mice, approximately 65% of the cells are eosinophils. Two mice from the asthmatic group developed a much lower percentage of eosinophils (40% and 20%) than the others and influence the variability among the group (see Fig.3.28a). In the treatment control group, all mice should have decreased eosinophils in their BALF, as the beneficial effect of dexamethasone is known. The percentage of lymphocytes and neutrophils stays under the 6% barrier. Like expected, the total number of BALF cells differs very significantly between the asthmatic mice (3.10^6 cells) and the naive mice (< 500,000 cells) as well as significantly between the asthmatic and the dexamethasone-treated mice (<1.10^6 cells, see Fig.3.28b). Interestingly, the number of eosinophils and neutrophils differ significantly and very significantly between the three control groups. Dexamethasone had a significant effect on the number of macrophages but not on the number of lymphocytes in the BALF compared to asthmatic mice. Unfortunately, the treatment with MAM-06.301 had no effect on the total BALF cell number or on the cellular BALF composition.

Effect of MAM.06.301 treatment on inflammation in lung tissue

The histological quantitative analysis of the mucus production in the airways confirmed the difference expected in the control groups. Asthmatic mice have a degree of mucus produced that differs highly significantly from the production in the naive and significantly from the mucus production in the dexamethasone-treated groups (see Fig.3.29a and Fig.3.31 A, B and C). Surprisingly, the twice a day treatment showed a significant effect on the mucus production compared to the asthmatic mice. Almost half of the mice in the once a day treatment group did not seem to
3.3 Effect of MAM-06.301 on an acute model of allergic asthma

respond to the treatment. Even if the extent of the eosinophilic infiltration in the asthmatic group differs very significantly from the naive group (see Fig.3.29b and Fig.3.32 A and B), half of the mice have a clearly higher degree of infiltration than the others. This might be the reason why the control treatment as well as both MAM.06.301 treatments have no significant effect on the peripheral eosinophilic infiltration, compared to the asthmatic mice (see Fig.3.32 C, D and E). Interestingly, the qualitative analysis based on the H&E staining testifies the decreased infiltration in the control-treated as well as in the MAM.06.301-treated groups (see Fig.3.33).

Effect of MAM-06.301 treatment on allergen-specific IgG1 antibodies

The naive mice have no IgG1 antibodies in their sera, but the control groups (asthmatic and dexamethasone-treated) do not differ significantly from it (see Fig.3.30). In this case, no statement is possible concerning the effect of the substance, as the control groups are not reliable.
Chapter 3 Results

(a) Percentage of individual leukocyte populations in BAL

(b) Total cell counts in BAL

**Figure 3.28** Effect of MAM-06.301 on inflammatory cells in BAL - Second experimental approach for acute asthma. Data are expressed as means ± SEM and represent the values of two experiments performed under the same conditions. Differences between groups respectively to the cell types were calculated with a two-tailed unpaired t-test. For total cell number, naive vs vehicle p=0.0066, vehicle vs dex. p=0.0173; for macrophage numbers, naive vs vehicle p=0.0250; for eosinophil numbers, naive vs vehicle p=0.0059, vehicle vs dex. p=0.0201; for neutrophils, naive vs vehicle p=0.0092, vehicle vs dex. p=0.0118; for lymphocytes, naive vs vehicle p=0.0247.
3.3 Effect of MAM-06.301 on an acute model of allergic asthma

(a) Mucus production in the airway

(b) Eosinophilic infiltration in the peripheral airways

**Figure 3.29** Effect of MAM-06.301 on mucus production and eosinophil number - Second experimental approach for acute asthma. Data are expressed as medians and represent the values of two experiments performed under the same conditions. Differences between groups were calculated with a Mann-Whitney test. For PAS, naive vs vehicle $p=0.0006$, vehicle vs dex. $p=0.0115$, vehicle vs Rx b.i.d $p=0.0271$; for LUNA, naive vs vehicle $p=0.0025$. 
Figure 3.30 Milk-specific IgG1 antibody level detected by ELISA - Second experimental approach for acute asthma. Data are expressed as means ± SEM and represent the values of two experiments performed under the same conditions.
3.3 Effect of MAM-06.301 on an acute model of allergic asthma

Figure 3.31 Lung histology in naive (A), asthmatic (B), dexamethasone-treated (C) and MAM-06.301-treated (D and E) mice. Representative photomicrographs of PAS whole-lung sections for goblet cells of the airway epithelium - Second experimental approach for acute asthma. Objective lens x200

Figure 3.32 Lung histology in naive (A), asthmatic (B), dexamethasone-treated (C) and MAM-06.301-treated (D and E) mice. Representative photomicrographs of LUNA whole-lung sections for eosinophil staining - Second experimental approach for acute asthma. Objective lens x400
Figure 3.33 Lung histology in naive (A), asthmatic (B), dexamethasone-treated (C) and MAM-06.301-treated (D and E) mice. Representative photomicrographs of H&E whole-lung sections for cell infiltration - Second experimental approach for acute asthma. Objective lens x40, x200 and x400
3.3 Effect of MAM-06.301 on an acute model of allergic asthma

3.3.3 Third experimental approach

In the third set of experiment the mice were administered MAM-06.301 intraperitoneally on five consecutive days, either once (Rx O.D) or twice daily (Rx b.i.d) but aerosolized with 2% milk solution twice a day on one single day for the same reasons than described in 3.1.3. The mice were assessed 72h after the last challenge and 24h after the last treatment (see Fig.2.7).

Effect of MAM-06.301 treatment on inflammatory cells in BALF

The percentage of eosinophils decreased to approximately 30% after the modifications brought to the disease induction protocol (see Fig.3.34a). Lymphocytes and neutrophils do not exceed 1%. Taken together these changes did not give the expected results that turned out to be rather disappointing. In fact, none of the groups, including the control groups, differ from each other concerning both their total BALF cell number and their BALF cell composition (see Fig.3.34b). A reason for this could be the very high variability among the groups, which would indicate that this disease setup is unreliable.

Effect of MAM.06.301 treatment on inflammation in lung tissue

The problem encountered in disease induction is reflected in the histological lung analysis. Even if the asthmatic group still differs very significantly from the naive mice in terms of mucus production (see Fig.3.35a and Fig.3.37 A and B) and eosinophilic infiltration (see Fig.3.35b Fig.3.38 A and B), dexamethasone and both MAM.06.301 treatments had no effect on these parameters (see Fig.3.37 and 3.38 C, D and E). As the necessary difference between positive and treatment control group does not appear, no conclusions can be drawn concerning an effect of the drug. In the H&E staining though, dexamethasone and to a lesser extent MAM.06-301 seem to decrease cell infiltration (see Fig.3.39).

Effect of MAM-06.301 treatment on allergen-specific IgG1 antibodies

Like expected, the IgG1 levels in the sera of all mice are lower than in the first setup. Naive mice have no IgG1, but similarly to the previous experiment, the antibody
Chapter 3 Results

levels in the mice of the control groups do not differ significantly from the naive mice (see Fig.3.36). The results are unsatisfying and due to problems to induce IgG1 in asthmatic mice with this model, we cannot speculate on an eventual effect of MAM-06-301 on the allergen-specific IgG1 production.

(a) Percentage of individual leukocyte populations in BAL

(b) Total cell counts in BAL

Figure 3.34 Effect of MAM-06-301 on inflammatory cells in BAL - Third experimental approach for acute asthma. Data are expressed as means ± SEM. Differences between groups respectively to the cell types were calculated with a two-tailed unpaired t-test.
3.3 Effect of MAM-06.301 on an acute model of allergic asthma

(a) Mucus production in the airway

(b) Eosinophilic infiltration in the peripheral airways

Figure 3.35 Effect of MAM-06-301 on mucus production and eosinophil number - Third experimental approach for acute asthma. Data are expressed as medians and represent the values of two experiments performed under the same conditions. Differences between groups were calculated with a Mann-Whitney test. For PAS, naive vs vehicle p=0.0092; for LUNA, naive vs vehicle p=0.0025
Figure 3.36 Milk-specific IgG1 antibody detection by ELISA - Third experimental approach for acute asthma
3.3 Effect of MAM-06.301 on an acute model of allergic asthma

**Figure 3.37** Lung histology in naive (A), asthmatic (B), dexamethasone-treated (C) and MAM-06.301-treated (D and E) mice. Representative photomicrographs of PAS whole-lung sections for goblet cells of the airway epithelium - Third experimental approach for acute asthma. Objective lens x200

**Figure 3.38** Lung histology in naive (A), asthmatic (B), dexamethasone-treated (C) and MAM-06.301-treated (D and E) mice. Representative photomicrographs of LUNA whole-lung sections for eosinophil staining - Third experimental approach for acute asthma. Objective lens x400
Figure 3.39 Lung histology in naive (A), asthmatic (B), dexamethasone-treated (C) and MAM-06.301-treated (D and E) mice. Representative photomicrographs of H&E whole-lung sections for cell infiltration - Third experimental approach for acute asthma. Objective lens x40, x200 and x400.
Chapter 4

Discussion

Asthma is a major health problem as demonstrated by its global increase in prevalence and incidence. Patients suffering from allergic asthma have recurrent episodes of coughing, wheezing, breathlessness and chest tightness. These symptoms are the consequence of chronic airways inflammation and airway hyperresponsiveness to allergens, leading to mucus hypersecretion and reversible airway obstruction. No cure exists for asthma. Currently, the most effective anti-asthmatic treatments available aim to relieve bronchoconstriction and control airway inflammation with a combination of glucocorticoids and beta-adrenoceptor agonists. However, there is a subpopulation of patients who are resistant to therapy. This project was focused on finding novel therapeutics for the treatment of allergic asthma.

4.1 Experimental mouse model of milk-induced allergic asthma

4.1.1 Usefulness of our mouse model

To test novel therapeutics, we used a model of allergic asthma developed in the laboratory using the allergen, milk. Milk is a useful antigen/allergen because it is a naturally occurring allergen, which generates an allergic response to a combination of proteins, mimicking allergic asthma in patients and it is much less expensive than purified proteins like ovalbumin. When sensitized and aerosol-challenged with milk, mice develop key features of allergic disease, including allergic lung inflammation, airway hypersecretion of mucus and production of antigen-specific IgE. Interestingly,
C57BL/6 (B6) mice respond more severely to milk, compared to the more "allergic" type BALB/c mouse strain. Thus, we adopted B6 mice to study the effects of two compounds with different mechanisms to inhibit allergic asthma. We measured airway and parenchymal inflammation, allergen-specific IgG1 antibodies in the serum and hypersecretion of mucus in lungs. We tested the compounds during the onset of allergic disease and then sought to determine whether the compounds could prevent an allergen-induced exacerbation\textsuperscript{50,87}. Tiotropium Bromide was tested on the acute and exacerbation asthma models and MAM-06.301 was tested during acute disease onset.

### 4.1.2 Effect on allergic airway inflammation

Mice were sensitized by 2 i.p injections of a solution of low-fat milk powder dissolved in PBS for primary allergen priming followed by respiratory tract allergen exposure with a nebulized milk solution. Mice were first challenged with four 2% milk solution aerosolizations but our results revealed that the extent of disease was too severe and thus, difficult to detect a treatment effect. Indeed, we observed a 75% infiltration of eosinophils and a similar level of parenchymal inflammation upon in situ histological analysis. This led us to modify the protocol to generate more moderate disease severity. We tested three different protocols (four 1% vs two 2% solution aerosolizations vs a single i.n instillation) and compared disease outcome (data not shown). Based on a moderate degree of inflammation, we adopted a “two-2%-solution” protocol. Our results confirmed the influence of changing the dose and frequency of aerosolizations on the extent of disease illustrated as an overall decrease of inflammation. While this protocol was promising, there was an unexpected increase in mouse-to-mouse variation that precluded its use. Using another protocol with “four-1%-solution”, we observed less variation and proceeded with the compound investigation. To study the effect of the compound on the prevention of disease exacerbation, we used recovered mice (in remission) and induced a disease relapse with four 1% milk challenges.
4.2 New insights on the effect of the novel compounds on inflammation processes

4.2.1 Investigations on a new indication for Tiotropium Bromide

Asthma was induced in mice by i.p injections of 10 mcg milk in 200 mcl 1x PBS on days 0 and 21, to prime an allergic immune response against milk. One week later, we aerosolized mice with either a 2 or 1 % milk solution. To test Tiotropium, we used intranasal instillation because our past experience, and that of others, has shown that it is as effective as intratracheal instillation. Intranasal instillation also has another advantage, which is that it is a minimally invasive method that is performed on conscious mice. Importantly, we used local administration of Tiotropium, as it is already available for inhalation therapy for other indications. Thus, Tiotropium was instilled directly to the site of inflammation in the lungs. To ensure consistent and accurate dosing, we administered the drug in a small volume of 50 mcl 1xPBS. The technique is simpler than inhalation exposure used in some studies, where meticulous calibration and high amount of drug represent considerable limits. Tiotropium is a muscarinic receptor antagonist, which we predicted would predominantly reduce AHR and mucus hypersecretion. An important property of Tiotropium is its kinetic selectivity for M1 and M3-receptors over M2-receptors. In fact, M1-receptors facilitate cholinergic neurotransmission and therefore enhance cholinergic bronchoconstriction, whereas M3-receptors on airway smooth muscle cells and glands mediate bronchoconstriction and mucus secretion. M2-receptors at cholinergic nerve endings inhibit the release of ACh and therefore act as feedback inhibitory receptors (autoreceptors). Taken together, Tiotropium’s long-acting targeted blockade of ACh release, suggest a beneficial effect on AHR and mucus production in asthma.

Effect of Tiotropium Bromide on AHR and mucus production  We found that Tiotropium was highly effective in the prevention of AHR in acute asthma, as expected because ACh-mediates bronchoconstriction. Tiotropium also inhibits airway M3-receptor-mediated mucus production, which is the reason we expected an effect on mucus hypersecretion. Surprisingly, mucus production remained unaffected
in our experiments. In contrast, there are studies in animal models showing that Tiotropium reduces allergen-induced mucus gland hypertrophy\textsuperscript{21} and airway smooth muscle remodeling\textsuperscript{59}. Inhaled anticholinergic agents are used for the treatment of chronic lung diseases in humans\textsuperscript{122}. Although it is not clear why mucus production was unaffected by Tiotropium. One possibility is that the complete dose did not reach the inflamed areas in the lungs and that perhaps nebulization would have been more effective because the particle size is smaller (3nm) and the drug would reach the more distant segments of the lungs. Another possibility is based on studies addressing the effect and mechanisms of action of Tiotropium that were either done in vitro on guinea pig trachea and human bronchi or in vivo on lung membranes of guinea pigs, dogs and humans\textsuperscript{6}. These other animals may respond differently than mice. A third possibility involves recent studies illustrating that there are differences in the neuronal nicotinic ACh receptor between mouse strains\textsuperscript{57,124}. In our studies, AHR experiments with Tiotropium were done with BALB/c mice because B6 mice do not develop AHR. It is possible that B6 mice are resistant to the M\textsubscript{3}-receptor blocking effect of Tiotropium and thus, we did not observe effects on mucus production. Future experiments using OVA, as an allergen in BALB/c mice will be done to further characterize the effect of Tiotropium on mucus production.

**Effect of Tiotropium Bromide on lung inflammation** Interestingly, 1 mg/kg of Tiotropium had a significant effect on airway inflammation during disease exacerbation. The overall severity and eosinophilia were reduced upon treatment. We expected that the drug would reduce inflammation during acute disease onset, but it was only able to reduce inflammation during exacerbation. These results support our previous results indicating that corticosteroids are able to inhibit chronic ongoing allergic asthma compared to acute disease\textsuperscript{88}. These results confirm that it is easier to treat ongoing or chronic allergic asthma than it is to prevent acute onset disease. Tiotropium is indicated for the treatment of COPD but not for asthma. There are interesting similarities between COPD and severe asthma, which has an inflammation pattern that differs from the mild to moderate asthma\textsuperscript{13,127}. A few common properties of both chronic lung diseases include increased numbers of neutrophils in the sputum, increased amount of CXCL8 and TNF, oxidative stress, low sensitivity to glucocorticoids, acute exacerbations and activation of NF\textsubscript{κ}B\textsuperscript{7,26,49,80,83}. These similarities have spurned the development of new therapeutic approaches\textsuperscript{44,95}. Our results support the idea that Tiotropium may be better at treating inflammation
4.2 New insights on the effect of the novel compounds on inflammation processes

in severe asthma, which is more similar to COPD. Tiotropium may have additional features that make it promising for the treatment for allergic asthma. Muscarinic receptor signaling appears to play a role in the pathophysiology of asthma and COPD\textsuperscript{60}. There is some suggestion that Tiotropium could inhibit airway remodeling and diminished lung function, in addition to its role as a bronchodilator\textsuperscript{44}. In a study in a chronic guinea pig model of asthma, Tiotropium was as effective as the steroid budenoside, in inhibiting airway remodeling\textsuperscript{21}. Recently, Tiotropium has been found to be effective for severe asthma with a non-eosinophilic phenotype\textsuperscript{86} in humans, which may explain the lack of effect on eosinophilic inflammation in the airways and lung tissue observed in our experiments. Previous studies have reported a relationship between airway remodeling and asthma severity\textsuperscript{76} and according to others, airway remodeling would explain the lack of response to therapy in some patients. By taking into account the important role of ACh in airway remodeling and the strong association between airway remodeling and severe asthma, it is conceivable that Tiotropium would stand the test in a chronic asthma model in future experiments. Tiotropium may be more potent when used in combination with anti-inflammatory agents. One study has shown that the anticholinergic drug glycopyrrolate acts synergistically with the PDE4 inhibitor rolipram and budesonide in inhibiting inflammatory mediators\textsuperscript{115}. In another study, the beta-adrenoceptor agonist carmoterol had an additive effect when combined with Tiotropium\textsuperscript{125}.

4.2.2 The natural component MAM-06.301 for a new application

We tested the effect of MAM-06.301, on acute onset allergic asthma using intraperitoneal injections because this route of administration had already been successfully used in other experiments. The mice were treated with 1 mg/kg of the drug, either once (O.D.) or twice a day (b.i.d). Control groups were treated with the same dose of the corticosteroid, dexamethasone, which is the best known treatment for allergic asthma in patients and in mouse models. Asthma is characterized by the expression of multiple genes for inflammatory proteins regulated by transcription factors. Several transcription factors are involved in asthmatic inflammation and lead to coordinate expression of multiple inflammatory mediators. Recent findings indicate that MAM-06.301 might inhibit NFκB\textsuperscript{108}, which has been associated with recruitment of eosinophils by eotaxin and activation of macrophages and epithelial
cells through iNOS (inducible form of nitric oxide synthase)\(^8\,70\,121\). MAM-06.301 may be therefore useful in the treatment of asthma associated with oxidative stress. MAM-06.301 had not yet been tested in allergic asthma, however, we hypothesized that it may attenuate disease via its anti-edematous and anti-inflammatory properties. Additionally, MAM-06.301 had been shown previously to exhibit anti-allergic properties in other disease models (personal communication, Dr. E. Prieschl-Grassauer).

**Effect of MAM-06.301 on mucus production and lung inflammation** Our results show an effect of MAM-06.301 on mucus secretion in peripheral airways and on inflammation. We detected decreased mucus secretion and inflammatory cell infiltration after administering both 7 and 10 doses. In contrast, 7 days of once a day treatment reduced eosinophilic infiltration whereas, 10 doses delivered in 5 days did not. An explanation could be that the drug acts better when given during a longer period of time. An explanation could be that the drug acts better when given during a longer period of time. Taken together, MAM-06.301 is able to decrease overall inflammation more rapidly, than the number of eosinophils, which may indicate a time-dependent effect of the drug on eosinophils. Moreover, the effect of MAM-06.301 may be even more relevant in a model, where acute inflammation is dampened and resembles more the degree of inflammation seen in humans (see 4.1.2). Nevertheless, our results give promising perspective for the therapeutic use of MAM-06.301 on mucus hypersecretion and inflammation in allergic asthma.

**Effect of MAM-06.301 on airway inflammation** MAM-06.301 exhibits anti-inflammatory properties in different disease models\(^23\,52\,53\,104\,120\). Surprisingly, MAM-06.301 treatment did not modulate airway inflammation. It is not clear why this anti-inflammatory drug was ineffective in the airways. However, there are several possible explanations. One possibility is that the systemic route of administration was ineffective because it did not reach high enough concentrations in the lungs. Because it is possible to deliver drugs locally in allergic asthma, the future plan in the lab is to attempt to use MAM-06.301 intranasally. A second explanation for the lack of response may be because the dose was insufficient. In this case, it is important to increase the dosing, by increasing either the dose or the frequency of administration.
4.3 Summary

**Effect of MAM-06.301 in allergic asthma** MAM-06.301 has other important effects that may be useful for the treatment of allergic asthma. It has been shown to regulate the cell cycle growth of colon cancer cells by inducing p21 (waf1/cip1)\(^{117}\). MAM-06.301 may act on lung epithelial cells and airway remodeling, which would potentially make it an interesting drug for chronic allergic asthma where airway remodeling causes lung damage. This effect of, MAM-06.301 may make it an ideal drug for its influence on hyaluronidase activity, which interferes with proteoglycan metabolism by bronchial fibroblasts that may contribute to AHR in asthma\(^{147}\). This is supported by a study illustrating that hyaluronic acid plays a role in the development of fibrotic changes in the lung of rats\(^{65}\). It would, therefore, be intriguing to investigate whether the anti-hyaluronidase activity of MAM-06.301\(^{52}\) can improve subepithelial fibrosis implicated in asthma\(^{1}\). MAM-06.301 also has anti-edema properties, which would be an additional useful adjuvant effect of the drug for allergic asthma\(^{123}\).

4.3 Summary

New insights and promising perspectives have been gained on the role of Tiotropium Bromide and MAM-06.301 in an experimental mouse model of allergic asthma. We used a milk-induced B6 mouse model of allergic asthma for evaluation of mucus production, lung inflammation and serum immunoglobulin. An ovalbumin-induced BALB/c mouse model of allergic asthma was used to evaluate methacholine-induced AHR after Tiotropium treatment. Our results indicate that Tiotropium Bromide efficiently prevents AHR development for up to 7 days, in a dose-dependent manner, inhibited lung inflammation during disease exacerbation but did not modulate mucus hypersecretion. Tiotropium Bromide is in clinical use for COPD and may be useful for allergic asthma. However, more experiments need to be done. Our investigations with MAM-06.301 show a beneficial effect on mucus production and lung inflammation with systemic treatment. Further experimentation is necessary for a full evaluation of this drug with titrated doses and local administration. In conclusion, these data offer optimistic perspectives on the findings of novel compounds for the treatment of allergic asthma.
A Acronyms

Ab    Antibody
ACh   Acetylcholine
Ag    Antigen
AHR   Airway Hyperreactivity
APC   Antigen Presenting Cells
Aqua Dest Distilled Water
ATP   Adenosine Triphosphate
BALF  Bronchoalveolar Lavage Fluid
cAMP  Cyclic Adenosine Monophosphat
CD4   Cluster of Differentiation 4
CD23  Cluster of Differentiation 23
CCL   Chemokine Ligand
CCR   Chemokine Receptor
COPD  Chronic Obstructive Pulmonary Disease
CVI   Chronic Venous Insufficiency
cys-LT Cysteinyl-Leucotriene
DALY  Disability-Adjusted Life Year
DCs   Dendritic Cells
EDRF  Endothelium-Dependent Relaxing Factor
ELISA Enzyme-Linked Immunoabsorbant
FOXP3 Forkhead Transcriptional Factor Box P3
GAGs  glycosaminoglycans
GATA3 GATA binding protein 3
GC    Glucocorticoid
HDAC-2 Histone Deacetylase-2
5-HT  Serotonin
IL    Interleukin
iNKT  invariant Natural Killer T cells
A Acronyms

IFN-γ Interferon-γ
LABA Long-Acting β-Adrenoceptor Agonists
LPS Lipopolysaccharide
mAChR Muscarinic Acetylcholine Receptor
MBP Major Basic Protein
NFAT Nuclear Factor
NFκB Nuclear Factor-κB
NO Nitric Oxide
PAF Platelet Activating Factor
PBS Phosphate Buffered Saline
PDE Phosphodiesterase
PECAM-1 Platelet Endothelial Cell Adhesion Molecule 1
PGs Prostaglandins
PKC-α Protein Kinase C-α
p38 MAPK p38 Mitogen-Activated Protein Kinase
RORγt Retinoid Acid Receptor-Related Orphan Receptor-γt
SCF Stem-Cell Factor
STAT Signal Transducer and Activator of Transcription
TLRs Toll-Like Receptors
TNF-α Tumour Necrosis Factor-α
TSLP Thymic Stromal Lymphopoietin
TSP-1 Thrombospondin-1
Tregs Regulatory T Cells
ULABA Ultra-Long-Acting β-Adrenergic
VCAM-1 Vascular Cell Adhesion Molecule 1
VLA-4 Very Late Antigen-4
WHO World Health Organization
# List of Figures

1.1 Structure of Tiotropium Bromide \((C_{19}H_{22}NO_4S_2Br\cdot H_20)\) .................................. 15
1.2 Structure of escin Ia \((C_{59}H_{86}O_{24})\) .......................................................... 17

2.1 Experimental setup - First experimental approach for acute asthma. . 24
2.2 Experimental setup - Second experimental approach for acute asthma . 24
2.3 Experimental setup - Third experimental approach for acute asthma . 25
2.4 Experimental setup - Memory model of allergic asthma . . . . . . . . . 25
2.5 Experimental setup - First experimental approach for acute asthma . 26
2.6 Experimental setup - Second experimental approach for acute asthma . 27
2.7 Experimental setup - Third experimental approach for acute asthma . 27

3.1 Effect of Tiotropium on inflammatory cells in BAL. . . . . . . . . . . . 36
3.2 Quantification of mucus production and eosinophil number. . . . . . . 37
3.3 Serum immunoglobulin. .......................................................... 38
3.4 Lung histology (PAS staining) .............................................. 39
3.5 Lung histology (LUNA staining) . . . . . . . . . . . . . . . . . . . . . . 39
3.6 Lung histology (H&E staining) .............................................. 40
3.7 Effect of Tiotropium on inflammatory cells in BAL. . . . . . . . . . . . 43
3.8 Quantification of mucus production and eosinophil number. . . . . . . 44
3.9 Serum immunoglobulin. .......................................................... 45
3.10 Lung histology (PAS staining) .............................................. 46
3.11 Lung histology (LUNA staining) . . . . . . . . . . . . . . . . . . . . . . 46
3.12 Lung histology (H&E staining) .............................................. 47
3.13 Effect of Tiotropium on inflammatory cells in BAL. . . . . . . . . . . . 50
3.14 Quantification of mucus production and eosinophil number. . . . . . . 51
3.15 Serum immunoglobulin. .......................................................... 52
3.16 Lung histology (PAS staining) .............................................. 53
3.17 Lung histology (LUNA staining) . . . . . . . . . . . . . . . . . . . . . . 53
List of Figures

3.18 Lung histology (H&E staining) ........................................ 54
3.19 Effect of Tiotropium on inflammatory cells in BAL. ............ 57
3.20 Quantification of mucus production and eosinophil number. .... 58
3.21 Serum immunoglobulin ............................................. 59
3.22 Lung histology (PAS staining) ....................................... 60
3.23 Lung histology (LUNA staining) ..................................... 60
3.24 Lung histology (H&E staining) ....................................... 61
3.25 Effect of MAM-06.301 on inflammatory cells in BAL. .......... 65
3.26 Quantification of mucus production, eosinophil number and cell infiltration ............................................. 66
3.27 Cytokine production .................................................. 67
3.28 Effect of MAM-06.301 on inflammatory cells in BAL. .......... 70
3.29 Quantification of mucus production and eosinophil number. .... 71
3.30 Serum immunoglobulin ............................................. 72
3.31 Lung histology (PAS staining) ....................................... 73
3.32 Lung histology (LUNA staining) ..................................... 73
3.33 Lung histology (H&E staining) ....................................... 74
3.34 Effect of MAM-06-301 on inflammatory cells in BAL. .......... 76
3.35 Quantification of mucus production and eosinophil number. .... 77
3.36 Serum immunoglobulin ............................................. 78
3.37 Lung histology (PAS staining) ....................................... 79
3.38 Lung histology (LUNA staining) ..................................... 79
3.39 Lung histology (H&E staining) ....................................... 80
Bibliography


Bibliography


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Bibliography


XIV


Bibliography


Marie Le Bras
Bräuhausgasse 11/34, A-1050 Vienna
T +43 650 9992500
email: marielebras@gmx.net

Born: 2 May 1983 (Sèvres, France)
Nationality: French

**UNIVERSITARY EDUCATION**

10/ 2002 - 07/ 2007 **Biology studies** with specialization on anthropology/ human genetics, University of Vienna

10/ 2001 - 07/ 2002 **Bioinformatics studies**, Ludwig - Maximilians - University of Munich, Germany

**PROFESSIONAL EXPERIENCE**

10/ 2007 - until now **Diploma thesis, Experimental Allergy Laboratory, Division of Immunology, Allergy and Infectious Diseases, Department of Dermatology, Medical University of Vienna**: Screening of novel therapeutics for the treatment of experimental allergic asthma in a mouse model.
- Induction of allergic asthma in mice
- Mouse dissection (collection of blood by heart puncture and of bronchoalveolar lavage fluid, extraction of spleen and lung)
- Differential cell counting of BAL cells using light microscopy
- Histological staining of lung sections embedded in paraffin (PAS, LUNA, H&E)
- Quantitative and qualitative analysis of histological data using light microscopy
- Serum extraction and analysis by ELISA

05/ 2007 - 08/ 2007 **Internship at the Institute of Biochemistry and Molecular Biology, University College of London, UK.** Investigation on the influence of GDF-15 on the regulation of hepcidin, a molecule with an important role in the regulation of iron metabolism.
- Cell culture (maintenance and propagation of human choriocarcinoma and hepatoma cell lines)
- RNA extraction from mouse placenta and RNA quantification using a spectrophotometer
- cDNA synthesis and real-time PCR

- DNA isolation from tumours embedded in paraffin
- DNA sequencing and computer-based analysis

02/ 2002 - 03/ 2002 **Internship at the Institute for Prophylaxis and Epidemiology, Ludwig Maximilian University of Munich, Germany.** Expression patterns of LIM kinase-1 and 2 and cofilin in platelets and endothelial cells; determination of phosphorylation state during platelet activation.
- Western Blot and protein detection using immunoblot and chemoluminescence
- Isolation of human umbilical vein endothelial cells and from plasmid
- Bacterial transfection
- Immunofluorescence

09/ 2001 **Internship at the Institute of Immunology, Ludwig-Maximilian University of Munich, Germany.** Mutation analysis of p53 in tumour cells.
- PCR
- Agarose gel electrophoresis
- SSCP analysis in SDS-PAGE
- DNA cloning in bacteria

**SCHOLARSHIP**

05/ 2007 - 08/ 2007 **Leonardo da Vinci Scholarship for internship placement abroad (EU Education Programme)**
Institute of Biochemistry and Molecular Biology, UCL, UK

**CONGRESS PARTICIPATION**

12/ 2007 **Annual Meeting of the Austrian Society for Allergology and Immunology (ÖGAI),** December 13 - 15, Alpbach in Tirol, Austria

09/ 2008 **Joint Annual Meeting of Immunology of the Austrian and German Societies (ÖGAI, DGfI),** September 3 - 6, Vienna, Austria
EDUCATION

1993 - 2001  
Lycée Jean Renoir, Munich, Germany  
German - French high school completed with: Baccalauréat scientifique (focus on mathematics, physics and natural sciences)

LANGUAGES

<table>
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COMPUTER SKILLS

Microsoft Office, Adobe Photoshop, Latex, JabRef, GraphPad Prism, Lucia G, SPSS

HOBBIES

Traveling, reading, diving, climbing, interior decoration

PRESENTATION

12/ 2007  
Meeting of the Physiological Society in Bristol  
Title: “A comparison of P2 receptor mRNA expression levels in the renal collecting duct in response to altered dietary sodium and in DOCA-induced hypertension.”
Authors: L. Yew-Booth\textsuperscript{2}, M. Le Bras\textsuperscript{2}, S. Balesaria\textsuperscript{2}, J. Marks\textsuperscript{2}, C. M. Turner\textsuperscript{2}, R. J. Unwin\textsuperscript{1}, S. S. Wildman\textsuperscript{1,2}