INTRODUCTION
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Asthma is one of the most significant among the health problems in the present world which reduces the work efficiency and quality of life remarkably causing immense damage to the national and international economy\(^1\). The clinical hallmarks of asthma are recurrent, episodic bouts of coughing, shortness of breath, chest tightness, and wheezing. It is physiologically characterized by hyperresponsiveness of the trachea and bronchi to various stimuli, in which the airway occasionally constricts, becomes inflamed, and is lined with excessive amounts of mucus causing minor wheezing to severe difficulty in breathing. In some cases, breathing may be so labored that an asthma attack becomes life-threatening\(^2\). For many patients, the disease has its roots in infancy, and both genetic factors (atrophy) and environmental factors (viruses, allergens, and occupational exposures) contribute to its inception and evolution\(^3\).

Asthma is a complex chronic inflammatory disease of the airways that involves the activation of many inflammatory and structural cells, all of which release inflammatory mediators that result in typical pathophysiological changes. Airway epithelial cells, smooth muscle cells, endothelial cells and fibroblasts are all capable of synthesizing and releasing an enormous variety of mediators which have more than one potent effect on airway inflammation\(^4\). Thus it can be understood that drugs affecting a single mediator are unlikely to be of substantial benefit.

Advances in this field have been greatly assisted by the development of potent and selective inhibitors that either block the inflammatory mediator receptors or inhibit mediator synthesis. Current asthma therapy involves relaxation of airway smooth muscle and inhibition of the underlying pulmonary inflammatory cells. However, available antiasthmatic agents such as anticholinergic agents, \(\beta_2\) selective adrenergic agonists, methyl xanthines, antihistamines, mast cell stabilizers, corticosteroids produce both these pharmacological effects inadequately and some are suitable only for symptomatic relief and most of them possess cardiotonic, CNS stimulatory, diuretic and other undesirable side effects\(^5\). This necessitates further investigation in this field to develop potent clinical candidates.
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Prevalence of Asthma

It is estimated from the executive summary of the GINA dissemination committee report that approximately 300 million people (5% of the global population or 1 in 20 persons) have asthma. Moreover, according to the World Health Organization, 255,000 people died of asthma in 2005 and asthma is the most common chronic disease among children. The prevalence of asthma has been rising worldwide over the past 50 years, especially in western countries. However, a wide variation of its prevalence across various countries has been observed.

Several surveys investigating children and adults in Eastern and Western Europe shows a marked difference in the prevalence of asthma depending upon the criteria used to define asthma. Two surveys conducted on British children 12 years of age between 1973 and 1988 showed that the prevalence of asthma increased from 5.6% to 10.3% in boys and from 2.7% to 7.9% in girls. The overall prevalence increased from 4.2% to 9.1%. In New Zealand, the prevalence of asthma increased from 26.2% to 34.0% between 1975 and 1989 among adolescents (12–18 years of age). From 1969 to 1982, the reported prevalence of ever having asthma among 11–13-year-old children in New Zealand increased from 7.1% to 13.5%. In Finland, men of conscription age (20 years) were studied during 1926 and 1939 and the prevalence remained steady between 0.02% and 0.08%. Even in 1981, the rate plateaued at 0.08%. However, by 1989, it had reached 1.79%. The increase in the prevalence of asthma was more in boys (2.8% in 1964 to 6.6% in 1989) than in girls (1.3% in 1964 to 3.8% in 1989) and it rose from 4.1% in 1964 to 10.2% in 1989 among schoolchildren of Aberdeen in the age group of 8–13 years. Age-specific rates of admission to hospital for asthma in all the age groups and both the sexes in England and Wales showed an increasing trend during the period 1976 to 1991–92. In England, from 1956–57 to 1968–69, the reported prevalence of currently diagnosed asthma among subjects in the age group of 4–18 years increased from 1.8% to 2.3%. A mixed longitudinal study of primary school children in England was conducted between 1973 and 1986. The results demonstrate that there is a true increase in morbidity. The prevalence of asthma in children 6–17 years of age was studied through a nationwide survey in the USA which shows a higher prevalence in boys than in girls, and the prevalence increased from 1963–1965 to...
1976–1980. No relationship between the socioeconomic status and asthma was seen. The prevalence of asthma ranged from 1.7% in 0–9-year-old children in urban Ethiopia to 9.4% in those in the age group of 60–69 years; from 1.1% in 0–9-year-old children to 3.3% in those in the age group of 60–69 years in rural areas. The prevalence was higher in urban (3.6%) than in rural areas (1.3%). A nationally representative sample of 0–17-year-old children in the USA was surveyed in 1981 and 1988. The results indicate that the prevalence of asthma is higher in boys than in girls (1981: boys 3.8%, girls 2.3%; 1988: boys 5.1%, girls 3.4%) and there was an increase in the prevalence during this period. Children born with normal weight (>2.5 kg) had a lower prevalence of asthma than those with low birth weight. Further, the risk of asthma in children from low-income families was high at both time points of the survey. The prevalence of asthma has been increasing for the past two to three decades in the industrialized countries of the West. The overall current prevalence of asthma is 8%–10% in the USA, and 20%–25% in the UK, Australia and New Zealand. In a study on students in the age group of 10–18 years in Chandigarh, 2.3% were diagnosed with asthma. The prevalence varied with age and the lowest prevalence was seen among those in the age group of 13–14 years. According to the National Family Health Survey-2 (NFHS-2) report the estimated prevalence of asthma in India is 2468 per 100,000 persons. The prevalence was higher in rural than in urban areas (2649 v. 1966). The prevalence among males was slightly higher (2561) than among females (2369). Among those below 15 years of age, asthma was seen in 950 per 100,000 persons. The prevalence rate was 2309 among those in the age group of 15–59 years, while it was 10,375 in those above 60 years of age. The prevalence of asthma in adult males (18 years and above) during 1995–97 was 3.94% in urban and 3.99% in rural areas. In females of the same age group, the prevalence was 1.27% in urban as well as rural areas. In earlier studies in the 1960s on adults (above 18 years of age), the prevalence of asthma in Delhi was 1.8% and 1.76% in Patna. Among adults in the age group of 20–45 years, no specific age-related pattern in the prevalence was seen in Mumbai. The study also revealed a ratio of 6.7 untreated cases to each treated case of asthma. A strong correlation of asthma with the family history was also seen. The prevalence of bronchial asthma in women from villages near Chandigarh city was 0.6% and it varied from a low of 0.2%
among LPG users to a high of 0.9% in those using stoves for cooking. However, 2.9% of those using a chullah for cooking had chronic bronchitis as compared to the overall prevalence of 1.9% in the community24.

Despite the scientific progress made over the past several decades toward improving our understanding of the pathophysiology of asthma, there is still a great need for improved therapies. Therefore, there is an unmet medical need for a new class of safe, effective oral drugs with a novel mechanism of action in asthma that targets specific pathophysiological mechanisms to prevent the progressive loss of lung function associated with airway remodelling and, in so doing, treats the underlying disease and not just the symptoms.

Pathophysiology of Asthma

As rational approach to the pharmacotherapy of asthma depends on an understanding of the disease's pathogenesis, there is an urgent need to identify the underlying basis of asthma, understand the complex genetic and environmental influences, and develop appropriate treatment strategies. Asthma is physiologically characterized by increased responsiveness of the trachea and bronchi to various stimuli and by widespread narrowing of the airways that change in severity1. Histologically, the airways of patients with irreversible or refractory asthma reveal subepithelial deposition of matrix proteins, increased airway smooth muscle cell mass, mucus gland hyperplasia, bronchial neovascularization, mucosal thickening from edema and cellular infiltration, and contraction of airway smooth muscle collectively termed airway remodeling (Fig. 1)25. Despite considerable research effort and clinical interest focused on airway remodeling in asthma, much remains unknown2.
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Fig. 1. Integrative model of the components of airway remodeling\textsuperscript{25}. The top figure represents a normal airway. The bottom figure depicts the histologic changes associated with asthma. In asthma, there exists airway inflammation, epithelial and goblet cell phenotype alterations, subepithelial fibrosis, excess mucus secretion, smooth muscle cell hypertrophy and hyperplasia and angiogenesis. Not depicted is the disruption of elastin fibers or the degenerative changes in cartilage.

The classic immunologic model shows that asthma is a disease mediated by reaginic (IgE) antibodies bound to mast cells in the airway mucosa (Fig. 2)\textsuperscript{2}. On reexposure to an antigen, antigen-antibody interaction on the surface of the mast cells triggers both the release of mediators stored in the cells' granules and the synthesis and release of other mediators. The agents which are responsible for the early reaction—immediate bronchoconstriction—include histamine, tryptase and other neutral proteases, leukotrienes C4 and D4, and prostaglandins. These agents diffuse throughout the airway wall and cause muscle contraction and vascular leakage. Other mediators are responsible for the more sustained bronchoconstriction, cellular infiltration of the airway mucosa, and mucus hypersecretion of the late asthmatic reaction that occurs 2–8 hours later. These mediators are thought to be cytokines characteristically produced by T-helper 2 (Th2) lymphocytes, especially GM-CSF and interleukins 4 (IL-4), IL-5, IL-9, and IL-13, which attract and activate eosinophils and stimulate IgE production by B lymphocytes\textsuperscript{26}. In particular, IL-4 promotes IgE synthesis in B cells,
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while IL-5 supports eosinophil survival\textsuperscript{27}. The Fig. 3 represents a simplified view of allergic inflammation in the airways\textsuperscript{27}. The etiology of airway inflammation, however, remains elusive, encompassing the interplay between genetic predisposition, environmental triggers and dysregulated immune responses\textsuperscript{1,2,25}.

The mechanisms underlying bronchial hyperreactivity are found to be related to inflammation of the airway mucosa. The agents that increase bronchial reactivity, such as allergen inhalation and infection with respiratory viruses, also cause airway inflammation. It is observed that the increase in bronchial reactivity induced by allergen inhalation is associated with an increase in both eosinophils and polymorphonuclear leukocytes in bronchial lavage fluid.

The most consistent difference in bronchial mucosal biopsies obtained from asthmatic and healthy subjects is an increase in the number of eosinophils found beneath the airway epithelium. Immunohistochemical staining shows increased levels of eosinophil cationic protein, indicating activation of the cells. The number of eosinophils in expectorated sputum or in fluid lavaged from the lungs correlates roughly with the degree of bronchial hyperreactivity. Eosinophil products have in turn been shown to cause epithelial sloughing and an increase in contractile responsiveness of airway smooth muscle\textsuperscript{2,26}. The products of other cells in the airways, such as lymphocytes, macrophages, mast cells, sensory nerves, and epithelial cells, have also been shown to alter airway smooth muscle function, so a specific antagonist to a single mediator or class of mediators might not prove fully effective as asthma therapy\textsuperscript{4}.
Exposure to allergen causes synthesis of IgE, which binds to mast cells in the airway mucosa. After re-exposure to allergen, antigen-antibody interaction on mast cell surfaces triggers release of mediators of anaphylaxis: histamine, tryptase, prostaglandin D₂ (PGD₂), leukotriene C₄, and platelet-activating factor (PAF). These agents provoke contraction of airway smooth muscle, causing the immediate fall in FEV₁. Reexposure to allergen also causes the synthesis and release of a variety of cytokines: interleukins 4 and 5, granulocyte-macrophage colony stimulating factor (GM-CSF), tumor necrosis factor (TNF), and tissue growth factor (TGF) from T cells and mast cells. These cytokines in turn attract and activate eosinophils and neutrophils, whose products include eosinophil cationic protein (ECP), major basic protein (MBP), proteases, and platelet-activating factor. These mediators cause the edema, mucus hypersecretion, smooth muscle contraction, and increase in bronchial reactivity associated with the late asthmatic response, indicated by a fall in FEV₁ 2–8 hours after the exposure.
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Mucous hypersecretion, gland hyperplasia
Mast cells
Dasop trls
Vasodilation, angiogenesis
Cytokine
Chemokines
Th2 cells
Eosinophils
Leukotrienes
Prostaglandins
Purines
Histamine
Proteases
PAF
Protons etc.
Parasympathetic nerve activation and plasticity
Inflammation

Fig. 3. Simplified view of allergic inflammation in the airways. Asthma is an episodic narrowing of the bronchi thought to be caused by an underlying chronic inflammatory disorder. In allergic asthma, inhaled allergen initiates the inflammatory response by interacting with IgE bound to mast cells and basophils. This leads to a cascade of events involving other immune cells and resulting in the release of numerous inflammatory mediators into the interstitial space, where they influence the growth and function of cell types within the airway wall. The drugs available for the treatment of asthma are targeted at inhibiting the inflammatory responses and/or relaxing the bronchial smooth muscle. Letters denote the putative sites of action for the various classes of drugs used in treating asthma. β, β2 adrenergic agonists; cs, corticosteroids; l, leukotriene modifiers; m, muscarinic receptor antagonists; cr, cromolyn; t, theophylline; al, anti-IgE therapy. The sunburst (*) symbolizes an allergen.

The mechanisms for bronchial hyperreactivity / bronchoconstriction result not only from the direct effect of the released mediators but also from their activation of neural or humoral pathways (Fig. 4). Several studies of laboratory animals evident the
importance of neural pathways in asthma. It has been observed that the bronchospasm provoked in dogs by histamine can be greatly reduced by pretreatment with an inhaled topical anesthetic agent, by transection of the vagus nerves, and by pretreatment with atropine. Studies of asthmatic humans, however, have shown that treatment with atropine causes only a reduction in—not abolition of—the bronchospastic responses to antigens and to nonantigenic stimuli. While it is possible that activity in some other neural pathway (eg, the nonadrenergic, noncholinergic system) contributes to bronchomotor responses to nonspecific nonantigenic stimuli, their inhibition by cromolyn, a drug that appears to inhibit mast cell degranulation, suggests that both antigenic and nonantigenic stimuli may provoke the release from mast cells of mediators that stimulate smooth muscle contraction by direct and indirect mechanisms (Fig. 4).

From the above observations, it can be hypothesized that asthmatic bronchospasm results from a combination of release of mediators and an exaggeration of responsiveness to their effects—predicts that asthma may be effectively treated by drugs with different modes of action. Thus the bronchospasm in asthmatic patients could be reversed or prevented by drugs that reduce the amount of IgE bound to mast cells (anti-IgE antibody), prevent mast cell degranulation (cromolyn or nedocromil, sympathomimetic agents, calcium channel blockers), block the action of the products released (antihistamines and leukotriene receptor antagonists), inhibit the effect of acetylcholine released from vagal motor nerves (muscarinic antagonists), or directly relax airway smooth muscle (sympathomimetic agents, theophylline). The second approach for the treatment of asthma is aimed not just at preventing or reversing acute bronchospasm but at reducing the level of bronchial responsiveness and inflammation. As increased responsiveness appears to be linked to airway inflammation and because airway inflammation is a feature of late asthmatic responses, this strategy is implemented both by reducing exposure to the allergens that provoke inflammation and by prolonged therapy with anti-inflammatory agents, especially inhaled corticosteroids.
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Fig. 4. Mechanisms of response to inhaled irritants. The airway is represented microscopically by a crosssection of the wall with branching vagal sensory endings lying adjacent to the lumen. Afferent pathways in the vagus nerves travel to the central nervous system; efferent pathways from the central nervous system travel to efferent ganglia. Postganglionic fibers release acetylcholine (ACh), which binds to muscarinic receptors on airway smooth muscle. Inhaled materials may provoke bronchoconstriction by several possible mechanisms. First, they may trigger the release of chemical mediators from mast cells. Second, they may stimulate afferent receptors to initiate reflex bronchoconstriction or to release tachykinins (e.g., substance P) that directly stimulate smooth muscle contraction.

Recent studies suggest the association of genetic polymorphisms with the development of asthma. These associations, however, have not always been replicated in other populations, probably because of the complexity of the disease. Other potential
complicating factors include genotypic and phenotypic heterogeneity across study populations, as well as unrecognized differences in environmental interactions, described by Raby and Weiss\textsuperscript{28}. \textbf{Table 1} lists several genes and their association with asthma; however, a more in-depth discussion of specific “asthma-associated” genes is available\textsuperscript{29-35}.

\textbf{Table 1. Genes associated with asthma.}

<table>
<thead>
<tr>
<th>Gene</th>
<th>Association</th>
<th>Putative function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAM33</td>
<td>Asthma</td>
<td>Protease, cell proliferation</td>
<td>29</td>
</tr>
<tr>
<td>Tim-1</td>
<td>AHR, Th2 (murine)</td>
<td>Regulate APC function</td>
<td>30</td>
</tr>
<tr>
<td>TGFb</td>
<td>Asthma</td>
<td>TGFb promoter activity</td>
<td>31</td>
</tr>
<tr>
<td>PHF11</td>
<td>Severe asthma, IgE</td>
<td>Transcriptional control</td>
<td>32</td>
</tr>
<tr>
<td>DPP10</td>
<td>AHR</td>
<td>Peptidase</td>
<td>33</td>
</tr>
<tr>
<td>Nramp1 (Slc11a1)</td>
<td>Th2, IgE (murine)</td>
<td>Modulation of T-cell inflammatory responses</td>
<td>34</td>
</tr>
</tbody>
</table>

\textbf{Overview of Available Antiasthmatics}

Current pharmacotherapy of asthma involves relaxation of airway smooth muscle (bronchodilators) and inhibition of the underlying pulmonary inflammatory cells (anti-inflammatory agents). These drugs can be classified into three groups: (a) Long term control medications − taken prophylactically to control asthma symptoms over a long period of time; (b) Quick-relief medications − also called “rescue” medication and are taken for immediate relief for asthma symptoms (SOS); (c) Anti-IgE monoclonal antibodies − taken prophylactically to avoid the onset of an asthmatic attack. These three groups comprise six various classes of therapeutic agents that are presently indicated for asthma treatment: $\beta$ adrenergic receptor agonists, glucocorticoids, leukotriene inhibitors, mast cell stabilizers, methylxanthines, and inhibitors of immunoglobulin E (IgE)\textsuperscript{4,5,27}. 
**β₂ Adrenergic Receptor Agonists**

These drugs are generally used by direct inhalation to the airways. The agonists can be classified as short- or long-acting. Short-acting agonists are used only for symptomatic relief of asthma, whereas long-acting agonists are used prophylactically in the treatment of the disease.

The mechanism of the antiasthmatic action of β adrenergic receptor agonists involves the direct relaxation of airway smooth muscle and consequent bronchodilation. It has been observed that human bronchial smooth muscle contains a large numbers of β₂ adrenergic receptors and stimulation of these receptors activates the G\(_5\)-adenylyl cyclase–cyclic AMP pathway with a consequent reduction of in smooth muscle tone. β₂ Adrenergic receptor agonists also increase the conductance of Ca\(^{2+}\)-sensitive K\(^+\) channels in airway smooth muscle, leading to membrane hyperpolarization and relaxation.

The β₂ adrenergic receptors are also present on cell types in the airways other than bronchial smooth muscle. In particular, stimulation of β₂ adrenergic receptors are found to be associated with the inhibition of the function of numerous inflammatory cells, including mast cells, basophils, eosinophils, neutrophils, and lymphocytes. In general, stimulating β₂ adrenergic receptors in these cells increase intracellular cyclic AMP, ultimately inhibiting the release of inflammatory mediators and cytokines.

Long-term exposure to β₂ agonists may desensitize some of these receptor-response pathways; thus, there is little evidence that these drugs, used chronically, reduce airway inflammation. The mechanism underlying the extended duration of action of salmeterol appears related to its high lipophilicity. After binding the receptor, the less lipophilic, short-acting agonists are removed rapidly from the receptor environment by diffusion in the aqueous phase, while salmeterol persists in the membrane and only slowly dissociates from the environment of the receptor.

Due to lack of β₂-receptor selectivity, β₁-mediated cardiac effects, short duration of action and α-agonistic activity (bronchoconstriction), ephedrine and epinephrine are not a drug of choice for asthma. Epidemiological studies have suggested that episodes of increased asthma mortality in the 1940s were accompanied due to the use of ephedrine and epinephrine as antiasthmatics. Efforts were made to induce specificity towards β₂-
receptor which resulted in the introduction of isoprenaline (isoproteranol), a derivative with a bulkier isopropyl group at the nitrogen. Its action on α-receptor was nullified but trachycardial effects remained. Attempts made to minimize the trachycardial effects and to further improve selectivity towards β2-receptor resulted in terbutaline, metaproterenol, salbutamol and salmeterol which became some of the most widely used antiasthmatics. Metaproterenol is a moderately selective β-agonist with longer duration of action that can be administered by inhalation for the treatment of asthma. The β2-Selective agonists (terbutaline, salbutamol and salmeterol) are effective bronchodilatory agents with no cardiac side effects. These drugs have longer duration of action and used for the prophylaxis because of slow onset and long half-life period. Asthmatics do not develop tolerance to β2-agonist as normal subjects do. Traditionally, there has been a reluctance to use β2 agonists due to severe side effects like tachycardia and hypokalemia (due to stimulation of K+ entry into skeletal muscle).
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Mast Cell Stabilizers

It has been observed that human mast cells release a variety of preformed and newly generated mediators on activation either in vitro or in vivo\textsuperscript{36,37} (Table 2,3). Among the most important, both quantitatively and pathophysiologically (as potential mediators of asthma) are histamine, sulfidopeptide, leukotrienes (leukotrienes C\textsubscript{4}, D\textsubscript{4} and E\textsubscript{4}) and prostaglandin D\textsubscript{2}. These mediators are responsible for three major signs of asthma to a significant extent such as smooth muscle contraction, mucus hypersecretion and edema formation. Clinical studies\textsuperscript{16,17,38} suggest that mast cell proteases such as chymase, may also participate in the control of the last two processes. Several mast cell mediators may be more general amplifiers of the inflammatory response\textsuperscript{4,5,27}. For example, platelet-activating factor (PAF) and leukotriene B\textsubscript{4} have potent chemotactic and cell activating properties. The development of hyperreactivity may be related to genometric factors such as the thickness of the bronchial walls, edema formation in either the airway wall or the surrounding adventitia, an intrinsic abnormality of smooth muscle, neural reflexes, either local or involving the central nervous system or the effect of inflammatory cells and mediators, with concomitant epithelial damage\textsuperscript{39}.

The immunoglobin E (IgE) is found to be the major link between environmental agents for example pollen or house dust, mites and release of mediators from mast cells. The most important feature of IgE is its unique ability to bind with high affinity to receptors on mast cells and basophils. The factor which is important in the generation of allergy is the formation of antigen-specific IgE and fixing of this to mast cell receptors for IgE. The initial access of antigen permits it to interact with specific receptors on β-lymphocytes and T-lymphocytes, which leads to the production of IgE secretory plasma cells and suppressor cells which limit IgE production from stimulated β-cells respectively. In the pathogenesis of allergic reactions in case of rat and man, IgE is the principle antibody involved and in other species, such as guinea pig, subclasses of IgE are involved\textsuperscript{40}.

Cromolyn sodium (disodium cromoglycate) and Nedocromil sodium are the two main drugs that inhibit the release of inflammatory mediators from bronchial mast cells; reverse the increased functional activation in leukocytes obtained from the blood of
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asthmatic patients; suppress the activating effects of chemotactic peptides on human neutrophils, eosinophils, and monocytes; parasympathetic and cough reflexes; and inhibit leukocyte trafficking in asthmatic airways. The main use of cromolyn and nedocromil is to prevent asthmatic attacks in individuals with mild-to-moderate bronchial asthma. These agents are ineffective in treating ongoing bronchoconstriction. When inhaled several times daily, cromolyn inhibits both the immediate and the late asthmatic responses to antigenic challenge or to exercise. With regular use for >2–3 months, bronchial hyperreactivity is reduced, as measured by response to challenge with histamine or methacholine. Nedocromil is approved for use in asthmatic patients 12 years of age and older; cromolyn is approved for all ages.

<table>
<thead>
<tr>
<th>Class</th>
<th>Mediator</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preformed</td>
<td>Histamine</td>
<td>Vasodilation, Vasopermeability, itch, cough, bronchoconstriction, rhinorrhea</td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td>Adhesion molecular regulation</td>
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<td></td>
<td>Proteases</td>
<td>Vasodilation, Vasopermeability</td>
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<td></td>
<td>Heparin</td>
<td>Bronchoconstriction</td>
</tr>
<tr>
<td>Lipid-derived</td>
<td>LTC₄</td>
<td>Vasodilation, Vasopermeability, bronchoconstriction</td>
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<tr>
<td></td>
<td>LTB₄</td>
<td>Leukocyte chemotaxis</td>
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<tr>
<td></td>
<td>PGD₂</td>
<td>Vasodilation, Vasopermeability, bronchoconstriction, mucus secretion</td>
</tr>
<tr>
<td></td>
<td>PAF</td>
<td>Bronchoconstriction, Leukocyte chemotaxis</td>
</tr>
</tbody>
</table>

Table 2. Mast cell mediators of inflammatory processes.

Cromolyn sodium

Nedocromil sodium
Introduction

Cytokine TNF-α
IL-1 Broad promotion of inflammation
IL-3 Mast Cell Division
IL-4 Mast Cell Division, β lymphocyte immunoglobulin class switching to produce IgE
II-5 Eosinophil differentiation and chemotaxis
II-6 Lymphocyte growth and differentiation
II-8 Leukocyte chemotaxis
GM-CSF Stimulates neutrophils, eosinophils and macrophages
MIP-1α Monocytes, T lymphocyte, Eosinophil chemotaxis

Table 3. Pathophysiologic changes associated with mast cell mediators.

<table>
<thead>
<tr>
<th>Pathophysiologic event</th>
<th>Mast Cell Mediators</th>
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<tr>
<td>Smooth muscle contraction</td>
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<td></td>
<td>Leukotrienes C_4/D_4/E_4</td>
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<tr>
<td></td>
<td>Prostaglandins D_2</td>
</tr>
<tr>
<td></td>
<td>Thromboxane A_2</td>
</tr>
<tr>
<td></td>
<td>Leukotriene B_4*</td>
</tr>
<tr>
<td></td>
<td>Platelet-activating factors *</td>
</tr>
<tr>
<td>Mucus hypersecretion</td>
<td>Histamine</td>
</tr>
<tr>
<td></td>
<td>Leukotrienes C_4/D_4/E_4</td>
</tr>
<tr>
<td></td>
<td>Prostaglandins D_2</td>
</tr>
<tr>
<td></td>
<td>Chymase*</td>
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**Introduction**

<table>
<thead>
<tr>
<th>Increased vascular permeability (Edema formation)</th>
<th>Histamine</th>
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<tbody>
<tr>
<td></td>
<td>Leukotrienes $C_4/D_4/E_4$</td>
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<td></td>
<td>Prostaglandins $D_2$</td>
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<tr>
<td></td>
<td>Thromboxane $A_2$</td>
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<td>Leukotriene $B_4^*$</td>
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<td></td>
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<td>Chymase $^#$</td>
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<table>
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<td>Prostaglandins $D_2^*$</td>
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<td></td>
<td>Histamine $^*$</td>
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<td>Heparin</td>
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<td>Tryptase</td>
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<tr>
<th>Airway hyperreactivity</th>
<th>Leukotrienes $C_4/D_4/E_4$</th>
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<tr>
<td></td>
<td>Platelet-activating factors $^*$</td>
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<tr>
<td></td>
<td>Leukotriene $B_4^#$</td>
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<tr>
<td></td>
<td>Tryptase $^#$</td>
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* Mediator is relatively weak, in producing these effects or produces these effects indirectly.

# Mediator has been shown to have this property in animals but not in human systems.

**Glucocorticoids**

Systemic glucocorticoids are used to treat severe chronic asthma or severe acute exacerbations of asthma. The development of aerosol formulations significantly improved the safety of glucocorticoid treatment, allowing it to be used for moderate asthma. Asthmatic subjects who require inhaled $\beta_2$ adrenergic agonists four or more times weekly are viewed as candidates for inhaled glucocorticoids. Glucocorticoids do not directly relax airway smooth muscle and thus have little effect on acute bronchoconstriction. Their anti-inflammatory effects in asthma include modulation of cytokine and chemokine production; inhibition of eicosanoid synthesis; marked inhibition
of accumulation of basophils, eosinophils, and other leukocytes in lung tissue; and decreased vascular permeability. Because of their profound and generalized anti-inflammatory actions, glucocorticoids are the most effective drugs used in the treatment of asthma.

A major advance in asthma therapy was the development of inhaled glucocorticoids that targeted the drug directly to the relevant site of inflammation. These formulations greatly enhance the therapeutic index of the drugs, substantially diminishing the number and degree of side effects without sacrificing clinical utility. There are five glucocorticoids available for inhalation therapy: beclomethasone dipropionate, triamcinolone acetonide, flunisolide, budesonide, and fluticasone propionate; all are effective in controlling asthma at the appropriate doses. Inhaled glucocorticoids are used prophylactically to control asthma rather than acutely to reverse asthma symptoms. While inhaled glucocorticoids in asthma are effective, local and systemic adverse effects remain a concern (Table 4). Inhaled glucocorticoids have extremely low oral bioavailability owing to extensive first-pass metabolism by the liver and reach the circulation almost exclusively by absorption from the lung. In contrast to the beneficial effects on asthma, which plateau at ~1600 µg/day, the risk of adverse effects continues to increase at higher doses. Oropharyngeal candidiasis and, more frequently, dysphonia can be encountered.

Systemic glucocorticoids are used for acute asthma exacerbations and chronic severe asthma. Substantial doses of glucocorticoids (e.g., 40–60 mg prednisone or equivalent daily for 5 days; 1–2 mg/kg/day for children) often are used to treat acute exacerbations of asthma. Although an additional week at somewhat reduced dosage may be required, the steroids can be withdrawn once control of the symptoms by other medications has been restored; any suppression of adrenal function dissipates within 1–2 weeks. More protracted bouts of severe asthma may require longer treatment and slower tapering of the dose to avoid exacerbating asthma symptoms and suppressing pituitary/adrenal function. Previously, alternate-day therapy with oral prednisone was employed commonly in persistent asthma. Now, most patients with asthma are better treated with inhaled glucocorticoids. The adverse effects of systemic administration of glucocorticoids are severe, but treatment for brief periods (5–10 days) causes relatively
little dose-related toxicity. The most common adverse effects during a brief course are mood disturbances, increased appetite, impaired glucose control in diabetics, and candidiasis. However, development of aerosol formulations has led to the reconsideration of the therapeutic role of corticosteroids in asthma.

| **Table 4.** Potential adverse effects associated with inhaled glucocorticoids. |
|-----------------|-----------------------------|
| **Adverse Effect** | **Risk** |
| Hypothalamic-pituitary-adrenal axis suppression | No significant risk until dosages of budesonide or beclomethasone increased to >1500 mg/day in adults or >400 mg/day in children |
| Bone resorption | Modest but significant effects at doses possibly as low as 500 mg/day |
| Carbohydrate and lipid metabolism | Minor, clinically insignificant changes occur with dosages of beclomethasone >1000 mg/day |
| Cataracts | Anecdotal reports, risk unproven |
| Skin thinning | Dosage-related effect with beclomethasone dipropionate over a range of 400 to 2000 mg/day |
| Purpura | Dosage-related increase in occurrence with beclomethasone over a range of 400 to 2000 mg/day |
| Dysphonia | Usually of little consequence |
| Candidiasis | Incidence <5%, reduced by use of spacer device |
| Growth retardation | Difficult to separate effect of disease from effect of treatment, but no discernible effects on growth when all studies are considered |
**Introduction**

Leukotriene modifiers

The leukotriene-modifying drugs act either as competitive antagonists of leukotriene receptors or by preventing the synthesis of leukotrienes by inhibiting 5-lipoxygenase enzyme which catalyzes the formation of leukotrienes from arachidonic acid.

Leukotriene (LT) modifiers are long-term medications which have entered the clinical practice during 1996-97. Their development is an example of rational drug design following the elucidation of LT structures and the subsequent confirmation of their pathophysiological role as inflammatory mediators in asthma.

Cysteinyl leukotrienes (CysLTs) include leukotriene C4 (LTC4), leukotriene D4 (LTD4), and leukotriene E4 (LTE4). All the CysLTs are potent constrictors of bronchial smooth muscle. On a molar basis, LTD4 is ~1000 times more potent than is histamine as a bronchoconstrictor. The receptor responsible for the bronchoconstrictor effect of leukotrienes is the CysLT1 receptor. Although each of the CysLTs is an agonist at the CysLT1 receptor, LTE4 is less potent than either LTC4 or LTD4. Zafirlukast and montelukast are selective high-affinity competitive antagonists for the CysLT1 receptor. Inhibition of CysLT-induced bronchial smooth muscle contraction is likely involved in the therapeutic effects of these agents to relieve the symptoms of asthma.

The formation of leukotrienes depends on lipoxygenation of arachidonic acid by 5-lipoxygenase. Zileuton is a potent and selective inhibitor of 5-lipoxygenase activity and thus blocks the formation of all 5-lipoxygenase products. Thus, in addition to inhibiting the formation of the Cys-LTs, zileuton also inhibits the formation of leukotriene B4 (LTB4), a potent chemotactic autacoid, and other eicosanoids that depend on leukotriene A4 (LTA4) synthesis. Logically, the therapeutic effects of a 5-lipoxygenase inhibitor would include all those observed with the CysLT-receptor antagonists, as well as other effects that may result from inhibiting the formation of LTB4 and other 5-lipoxygenase products.
There are few adverse effects directly associated with inhibition of leukotriene synthesis or function. This is likely due to the fact that leukotriene production is limited predominantly to sites of inflammation. In large clinical studies the adverse-effect profiles of these drugs were similar to that observed with placebo treatment. Very rarely, patients taking these drugs develop systemic eosinophilia and a vasculitis with features similar to Churg-Strauss syndrome27.

Most clinical trials with these drugs have studied patients with mild asthma who were not taking glucocorticoids. In general, the studies show a modest but significant improvement in pulmonary function and a decrease in symptoms and asthma exacerbations. For those who respond to antileukotriene therapy, the National Heart, Lung, and Blood Institute recognizes these drugs as alternatives to low-dose inhaled steroids for control of mild chronic asthma. This class of drugs is not indicated for rapid bronchodilator therapy; thus, patients are instructed to have short-acting β adrenergic receptor agonists available as rescue medication. Montelukast and zafirlukast are effective with once- or twice-daily treatment, respectively. In contrast, zileuton is taken 4 times a day. Hepatic transaminases should be monitored in patients beginning zileuton therapy to guard against the potential of liver toxicity27.
Methyl xanthines

The xanthine derivatives caffeine, theobromine and theophylline are closely related alkaloids isolated from various plant sources. Methyl xanthines have been used in the treatment of asthma since 1930. Several derivatives are available, but none appear to be better than theophylline though enprofylline (3-propylxanthine) may be more potent. Many xanthine salts are available such as aminophylline which has increased solubility at neutral pH, rendering it useful for intravenous administration. Major therapeutic actions include relaxation of bronchial smooth muscle, decrease in mast cell mediator release, increase in mucociliary clearance and prevention of micro vascular leakage. All these actions are due to inhibition of phosphodiesterase enzyme (PDE), thereby increasing intracellular c-AMP levels, direct and indirect effects on the intracellular calcium concentration; increase in uncoupling of intracellular calcium with muscle contractile elements and antagonism of adenosine receptors. Combination therapy of mixtures of theophylline and β2-agonists were usually preferred. Side effects due to over dosage include headache, restlessness, increased secretion, diuresis, high concentration, convulsions and cardiac arrhythmias.4,5,27

Since there is no ideal treatment for asthma, there is a need to develop drugs which can affect a wide range of mediators which posed a challenge to researchers to develop innovative strategies. It has been observed that adenosine plays an important role in mediating bronchial constriction and pulmonary inflammation by interacting with its different G-protein coupled receptors: A1, A2A, A2B, A3 adenosine receptors (ARs)
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which are evidenced to be found in a number of inflammatory cell types including mast
cells, eosinophils, lymphocytes, neutrophils and macrophages. Therefore, it is
suggested that blockade of A₁, A₂b, A₃ ARs may be a valuable approach for the
treatment of asthma and chronic obstructive pulmonary disease (COPD)⁴¹.

Adenosine Receptors (AR): Promosing Targets for the Development of Novel
Therapeutics and Diagnostics for Asthma

Adenosine is an endogenous nucleoside consisting of purine base, adenine in
glycosidic linkage with the sugar ribose. This purine nucleoside adenosine is normally
present in human tissues at low concentrations, but in response to metabolic stress,
such as that encountered in the course of inflammatory events or during tissue hypoxia,
a rapid increase in adenosine tissue levels takes place⁴². Once generated, adenosine
elicits its biological activities by interacting with at least four adenosine receptor
subtypes belonging to a G-protein-coupled-receptor family: A₁, A₂a, A₂b and A₃
adenosine receptors⁴³. A role for adenosine in pulmonary disease was first suggested in
the late 1970s when it was found that adenosine and related synthetic analogues were
potent enhancers of IgE-dependent mediator release from isolated rodent mast cells⁴⁴.
A few years later, adenosine administered by inhalation was shown to be a powerful
bronchoconstrictor of asthmatic but, importantly, not of normal airways⁴⁵. Further work
showed that both allergic and non-allergic asthmatics responded in a similar way and
that the effect was also seen with adenosine 5′-monophosphate (AMP), ADP and
ATP⁴⁶-⁴⁸. In addition to its well-known effect as a bronchoconstrictor, a growing body of
evidence has emphasized the importance of adenosine in the initiation, progression and
control of chronic inflammation and remodeling of the airways⁴⁹,⁵⁰. This body of
evidence is supported by the following reported findings.

(a) In asthmatics adenosine levels are elevated in bronchoalveolar lavage (BAL)
fluid⁵¹, in the circulation following allergen inhalation⁴⁶, and in exhaled breath
condensate in patients with asthma⁵².

(b) Adenosine given by inhalation causes a dose-dependent bronchoconstriction in
subjects with asthma⁴¹,⁴⁵,⁵³.
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(c) Inhalational challenge with adenosine monophosphate (AMP), which is metabolized locally by the ectonucleotidase 5'-nucleotidase to adenosine, increases the release of leukotrienes and other bronchoconstrictive mediators in asthmatics\textsuperscript{54}.

(d) Adenosine enhances mast-cell allergen-dependent activation\textsuperscript{55}.

(e) Treatment with dipyridamole, a blocker of adenosine reuptake, significantly enhances the bronchoconstrictor response to inhaled adenosine in subjects with asthma\textsuperscript{56}.

(f) The sensitivity of airways to adenosine and AMP more closely reflects an inflammatory process and the phenotype for allergic asthma than the sensitivity of airways to other known inhalational bronchoprovocative agents, such as methacholine and histamine\textsuperscript{57-60}.

The above observations indicate that adenosine is likely to play an important role in asthma and COPD through interaction with specific cell-surface purinoreceptors. Expression of the four identified adenosine receptors has been shown in a large number of proinflammatory and structural cells and recently in the peripheral lung parenchyma of patients with COPD\textsuperscript{61}.

Adenosine Receptors (ARs)

These receptors belong to the superfamily of G-protein-coupled receptors and are characterized by seven-transmembrane-spanning α-helical domains with an extracellular amine terminus and a cytoplasmic carboxy terminus. Receptor subtypes are distinguished based on their affinity for adenosine, pharmacological profiles, G-protein coupling and signaling pathways, and genetic sequence. The physiological effects of adenosine are mediated by intracellular signaling processes that are specific to the receptor subtype and the type of cell. The adenosine A\textsubscript{1} receptor (A\textsubscript{1}AR) is coupled to the pertussis toxin (PTX)-sensitive inhibitory G proteins (G\textsubscript{i}) or G\textsubscript{o}. Activation of the A\textsubscript{1}AR can lead to the activation a number of effector systems, including adenylate cyclase (AC), phospholipase A\textsubscript{2}, phospholipase C (PLC), potassium channels, calcium channels, and guanylate cyclase\textsuperscript{43,62,63}. The primary changes in second messengers
associated with $A_1$AR activation are decreased production of cAMP or increased $Ca^{2+}$, depending on the effector system. Like the $A_1$AR, the adenosine $A_3$ receptor ($A_3$AR) is coupled to the PTX-sensitive $G_i$ protein and also to $G_q^{43}$. Activation of the $A_3$AR results in an inhibition of AC (leading to decreased cAMP) or stimulation of PLC and phospholipase $D^{64}$. The adenosine $A_{2A}$ receptor ($A_{2A}$AR) and adenosine $A_{2B}$ receptor ($A_{2B}$AR) share a relatively high homology and are coupled to $G_3^{43}$, leading to increased levels of cAMP. In addition, the $A_{2B}$AR has been shown to couple to $G_q^{65}$, thereby regulating intracellular $Ca^{2+}$ levels. In general, the $A_1$AR, $A_{2A}$AR and $A_3$AR subtypes have high affinity for adenosine, while the $A_{2B}$AR has a lower affinity$^{66}$.

**Adenosine Production and Metabolism**

The physiological effects of adenosine in asthma via its stimulation of cell-surface adenosine receptors (ARs) and subsequent downstream signaling pathways are a function of the local concentration of adenosine. Adenosine concentrations in unstressed cells and tissue are below 1 $\mu$M (estimates 10–100 nM); however, in metabolically stressed inflamed or ischemic tissues, adenosine levels may rise to 100 $\mu$M$^{66,67}$. Lower concentrations of adenosine (10–100 nM) activate the high-affinity $A_1$, $A_{2A}$, and $A_3$ ARs and high adenosine concentrations (10 $\mu$M) stimulate low-affinity $A_{2B}$ARs$^{66}$. Factors that determine the net effect of adenosine on specific cell and tissue function are AR expression and coupling to intracellular signaling pathways, all of which are tightly regulated in different tissues and cells. The local adenosine concentration at its receptor subtypes is determined by several processes, which include extracellular and intracellular adenosine generation, adenosine release from cells, cellular reuptake and metabolism (Fig. 5). These processes are closely intertwined and strictly regulated. For, example, under the hypoxic and inflammatory conditions encountered in asthmatic airways, the increased intracellular dephosphorylation of adenosine 5'-triphosphate (ATP) to adenosine by the cytosolic metabolic enzyme 5'-nucleotidase may be accompanied by a suppression of the activity of the salvage enzyme adenosine kinase, which prevents the rephosphorylation of adenosine to AMP$^{68}$. These processes lead to high adenosine concentrations inside the cell and the release of adenosine from the dephosphorylation of AMP into the extracellular space through nucleoside
transporters\textsuperscript{69,70}. The other major pathway that contributes to high extracellular adenosine concentrations during metabolic stress is release of adenine nucleotides (ATP, ADP and AMP) from inflammatory and injured cells. This is followed by extracellular degradation to adenosine by a cascade of ectonucleotidases, which include CD39 (nucleoside triphosphate diphosphohydrolase (NTPDase)) and CD73 (5'-ectonucleotidase)\textsuperscript{71-75}. Adenosine accumulation is limited by its catabolism to inosine by adenosine deaminase. Inosine is finally degraded to the stable end-product uric acid\textsuperscript{67,76}. Mechanisms of nucleotide release and metabolism, or adenosine release and metabolism, as well as transport mechanisms that account for the increased adenosine levels in exhaled breath condensate after exercise\textsuperscript{52}, in the circulation following allergen inhalation\textsuperscript{46}, and in BAL fluid (BAL adenosine concentration of 2.55 ± 0.50 μM in asthmatics versus 0.72 ± 0.16 μM in normals)\textsuperscript{51} in human asthmatics, are yet to be determined. There are several important cell types that are sources of extracellular adenosine. Neutrophils and endothelial cells release large amounts of adenosine at sites of metabolic distress, inflammation and infection\textsuperscript{77-80}. Activated leukocytes are a major source of extracellular adenosine\textsuperscript{81}. ADP released by platelets can be a significant source of adenosine after dephosphorylation\textsuperscript{83}. Under conditions of stress including infection, activated macrophages can also serve as a major source of extracellular adenosine via ATP metabolism. Bacterial lipopolysaccharide (LPS) augments the release of ATP from macrophages\textsuperscript{83}. Moreover, T-helper lymphocytes may be an important source of extracellular ATP. The presence of ecto-ATPase and antigen-triggered accumulation of extracellular ATP from T-helper cells has been reported\textsuperscript{84}. In addition to inflammatory cells, airway epithelial cells and other structural cells in the lung may be important sources of high levels of adenosine in the airways of human asthmatics\textsuperscript{85}. 

\textbf{Introduction}
Review on Adenosine-Induced Bronchoconstriction, Airway Inflammation, and Airway Remodeling

In asthmatics, adenosine produces bronchoconstriction, inflammation, and airway plasma exudation, which lead to airway obstruction. Moreover, by acting on ARs, adenosine induces the release of inflammatory mediators that are important in the pathogenesis of airway remodeling in asthmatics. In both humans and animals, adenosine induces increases in BHR in asthmatics but not normal subjects, both in vivo following inhalation and in vitro in small airways. Adenosine produces bronchoconstriction in airways by directly acting on ARs in bronchial smooth muscle cells or indirectly by inducing the release of preformed and newly formed mediators from mast cells, and by acting on ARs on airway afferent sensory nerve endings. Multiple mechanisms may be involved in adenosine-induced bronchoconstriction; for example, the effects of adenosine in asthmatic subjects are sensitive to muscarinic
receptor antagonists, suggesting that adenosine mediates obstruction indirectly, which would be consistent with the preclinical evidence that adenosine can activate afferent nerves in vivo. However, since muscarinic antagonists do not completely abolish bronchoconstriction in response to adenosine, it is plausible to conclude that the "atropine-resistant" component of this response is mediated by direct activation of airway smooth muscle and/or indirectly via mediators released from other cell types expressing these receptors.

Adenosine exposure through inhalation increases enhanced pause (Penh), a measure of airway resistance, in allergen-sensitized and -challenged mice. This increase in enhanced pause due to adenosine was reversed by theophylline with methacholine-mediated enhanced pause being unaffected, suggesting the involvement of ARs. This finding that adenosine-induced bronchoconstriction is mediated by ARs is supported by an earlier study in a rabbit model of allergic asthma, where adenosine-induced bronchoconstriction was blocked by theophylline. Following inhalation and its local metabolism to adenosine in the airway, AMP induced bronchoconstriction is attenuated by potent cyclooxygenase inhibitors, H₁ receptor and leukotriene receptor antagonists, suggesting that adenosine induces the release of prostaglandins, histamine and leukotrienes in the airways of asthmatics. Another study has shown that inhalation challenge with adenosine, but not methacholine, produces mild airway plasma exudation. Collectively, these effects of adenosine on airway nerves, contraction of bronchial smooth muscle, release of mast cell mediators, and airway edema produce airflow obstruction. Adenosine produces inflammation in airways in allergic animals and humans. Animals with increased adenosine concentrations in the lung (adenosine deaminase (ADA)-deficient mice) develop severe pulmonary inflammation, with airway accumulation of eosinophils and activated macrophages, mast cell degranulation, and mucus metaplasia in the airways—features similar to that found in asthmatic bronchi. Treatment of these mice with exogenous ADA to reduce adenosine concentrations results in the reversal of these asthmatic features.

In a mouse model of allergic asthma, inhalation of adenosine has also been shown to cause airway inflammation, as evidenced by an increased release of proinflammatory mediators from eosinophils and mast cells. Moreover, in human asthmatics, an
Adenosine-mediated inflammation is not limited to the lung; it also reaches the systemic circulation. In a recent report in a mouse model of asthma activities of eosinophilic peroxidase, myeloperoxidase and beta-hexosaminidase were increased not only in the lung but also in the systemic circulation of allergic mice exposed to adenosine aerosol\textsuperscript{104}. In human asthmatics, adenosine aerosol increases the release of neutrophil chemotactic factor in serum\textsuperscript{109}. Moreover, in a recent study it was demonstrated that adenosine-induced effects on urinary 9α, 11β-prostaglandin (PG) F\textsubscript{2} levels (a sensitive biomarker of mast cell degranulation) were enhanced during repeated low-dose allergen challenge in allergic asthmatics\textsuperscript{110}. These earlier findings in asthmatics were confirmed by a recent study showing an increase in plasma 9α, 11β-PGF\textsubscript{2} levels after adenosine challenge in asthmatics\textsuperscript{111}. These studies suggest that following inhalation, adenosine enhances the release of systemic inflammatory mediators from sensitized inflammatory cells. Thus, following inhalation, adenosine not only produces inflammation in the airways of asthmatics but it also induces a systemic inflammatory response that would, in turn, amplify the inflammation locally in the airways of asthmatics.

Adenosine in the lung may also be involved in the airway remodeling process\textsuperscript{85}. Pathogenic hallmarks of airway remodeling are mucous gland hyperplasia, subepithelial fibrosis, hypertrophy of bronchial smooth muscle, and angiogenesis\textsuperscript{85,112}. In a recent report, substantial angiogenesis in the tracheas of ADA-deficient mice were seen in association with high levels of adenosine\textsuperscript{113}. ADA replacement enzyme therapy in these mice resulted in a lowering of adenosine levels and reversal of tracheal angiogenesis. Moreover, in lung alveolar epithelial cells and lung fibroblasts, adenosine caused an induction of fibronectin (a matrix glycoprotein highly expressed in injured tissues that has been implicated in wound healing) mRNA and protein expression in a dose- and time-dependent manner\textsuperscript{114}. Furthermore, there appears to be a connection of IL-13 levels to high adenosine levels, ADA activity and airway remodeling\textsuperscript{115}. Studies in CC10 IL-13 Tg mice showed that IL-13 induced high levels of adenosine, inflammation, lung collagen content and subepithelial airway fibrosis and reduced ADA activity in the lung.
ADA therapy administered to these mice decreased adenosine levels, inflammation, and subepithelial airway fibrosis. Moreover, in ADA-deficient mice, IL-13 was strongly induced. These findings suggest that IL-13 and adenosine stimulate one another to amplify the pathway that contributes to airway inflammation, fibrosis, and remodeling. Similar findings were also seen in the lungs of mice overexpressing the Th2 cytokine IL-4.

Adenosine Receptors Signalling Pathways and their Role in Asthma

The above observations suggest a strong role for adenosine not only in the bronchoconstriction of allergic airways but also in the progression and amplification of airway inflammation and airway remodeling. The effects of adenosine as an important signaling molecule in asthma may depend not only on the bioavailability of the nucleoside but also on the expression, density, and affinity of ARs, which are known to be finely modulated by physiological and/or pathological conditions, signaling mechanisms, the local metabolism of adenosine, and the predominant inflammatory cell types in the asthma model, which may be species specific. Adenosine produces its effects in asthmatics by acting on membrane-bound extracellular ARs on target cells. Four subtypes of ARs (namely A1, A2A, A2B, and A3) have been cloned in humans, are expressed in the lung, and are all targets for drug development for human asthma. These receptors are heptaspanning-transmembrane G-protein-coupled receptors. Three of the AR subtypes (A1, A2A, and A2B) demonstrate 80–95% sequence homology across a wide evolutionary range of species. In contrast, the A3ARs demonstrate significant species variation. Signal transduction by the ARs varies; not only among the subtypes but also for a particular subtype between different cell sources. A1ARs were originally characterized as being coupled to pertussis-toxin-sensitive Gq-coupled signal transduction pathways, but in some cells they are directly associated with, and act through, ion channels. The A2AR subtypes (A2A and A2B) are typically coupled to Gs-linked signal transduction pathways. In some cells, A1AR receptor-mediated inhibition and A2A AR mediated stimulation of adenylate cyclase may coexist and their functions may be counterregulatory. A summary of the AR subtypes,
their signal transduction mechanisms, and selective agonists and antagonists is presented in Table 5.

Classically, AR signalling is thought to occur through inhibition or stimulation of adenylyl cyclase (also known as adenylate cyclase), although it is now apparent that other pathways, such as phospholipase C (PLC), Ca2+ and mitogen-activated protein kinases (MAPKs), are also relevant (Fig. 6).

Activation of the AiAR inhibits adenylyl cyclase activity through activation of pertussis toxin-sensitive G\textsubscript{i} proteins\textsuperscript{123,124} and results in increased activity of PLC\textsuperscript{125,126}. In cardiac muscle and neurons, AiARs can activate pertussis toxin-sensitive K\textsuperscript{+} channels, as well as K\textsubscript{ATP} channels, and inhibit Q-, P- and N-type Ca\textsuperscript{2+} channels\textsuperscript{1}. Coupling to K\textsuperscript{+} channels in supraventricular tissue is responsible for the bradycardiac effect of adenosine on heart function\textsuperscript{127}. In the heart, AiAR and A\textsubscript{2A}AR agonist induced preconditioning has been suggested to occur via modulation of p44/42 extracellular signal-regulated protein kinase (ERK) signalling\textsuperscript{128}.

Activation of the A\textsubscript{2A}AR increases adenylyl cyclase activity. G\textsubscript{s} seems to be the major G-protein associated with A\textsubscript{2A}ARs in the peripheral systems but not in the striatum, where A\textsubscript{2A}AR density is the highest. It has been shown that striatal A\textsubscript{2A}ARs mediate their effects predominantly through activation of G_olv\textsuperscript{129}, which is similar to G\textsubscript{s} and also couples to adenylyl cyclase. In rat tail artery, facilitation of noradrenaline release by activation of the A\textsubscript{2A}AR triggers the PLC and adenylyl cyclase pathways\textsuperscript{130}. Activation of the A\textsubscript{2A}AR also induces formation of inositol phosphates to raise intracellular calcium and activate protein kinase C in COS-7 cells via pertussis toxin-insensitive Ga\textsubscript{15} and Ga\textsubscript{16} proteins\textsuperscript{131}, which have limited tissue distribution and interact with most GPCRs.

The A\textsubscript{2B} AR is positively coupled to both adenylyl cyclase and PLC\textsuperscript{132–134}. Results indicate that the activation of PLC, through G\textsubscript{q} proteins, mediates many of the important functions of A\textsubscript{2B}ARs\textsuperscript{135,136}. Activation of the A\textsubscript{2B} AR by the non-selective agonist NECA increased inositol phosphate formation in human mast cell line HMC-1\textsuperscript{134}, which is not sensitive to cholera or pertussis toxin but is antagonized by the slightly A\textsubscript{2B} AR-selective antagonist enprofylline (3-propylxanthine)\textsuperscript{134}. The arachidonic acid pathway was also recently demonstrated to be involved in A\textsubscript{2B} AR activation\textsuperscript{137}. 

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The A3 AR couples to classical second-messenger pathways such as inhibition of adenylyl cyclase\textsuperscript{138}, stimulation of PLC\textsuperscript{139} and calcium mobilization\textsuperscript{140-143}. In cardiac cells, A3 AR agonists induce protection through the activation of KATP channels\textsuperscript{144}. RhoA–phospholipase D\textsubscript{1} signalling has been demonstrated to mediate the antiischaemic effect of A3 ARs\textsuperscript{145}. The WNT signalling pathway is involved in A3 AR agonist-mediated suppression of melanoma cells\textsuperscript{146}. In addition, like other ARs, the A3 AR couples to MAPK, which could give it a role in cell growth, survival, death and differentiation\textsuperscript{147,148}. An A3 AR agonist inhibits proliferation in A375 human melanoma cells via the phosphatidylinositol 3-kinase–protein kinase B–ERK1/2 pathway\textsuperscript{149}.

Phosphorylation and subsequent desensitization of ARs have been studied for all four subtypes. The rapidity of the desensitization depends on the subtype, with the A3 AR being more rapidly desensitized than the other subtypes\textsuperscript{63,64,150}. GPCR kinase-mediated mechanisms are thought to have a crucial role in the rapid desensitization of A\textsubscript{2A} and A\textsubscript{2B} ARs\textsuperscript{150}. Novel disease targets for selective adenosine receptor ligands are shown in Fig. 7.

Adenosine receptors have been described on a number of different cell types that are important in the pathophysiology of asthma, including dendritic, antigen-presenting cells, human airway epithelial and bronchial smooth muscle cells, lymphocytes, mast cells, eosinophils, neutrophils, macrophages, fibroblasts and endothelial cells\textsuperscript{120-122}. Activation of ARs on these different cell types is responsible for inducing the release of mediators and cytokines, leading to BHR, inflammation, edema, and airway remodeling. Activation of ARs on afferent sensory airway nerves contributes to BHR in asthma\textsuperscript{80}. The contributions of the different AR subtypes to the pathophysiology of asthma are presented in Fig. 8. Furthermore, the targeting of ARs with selective agonists or antagonists as therapeutic strategies in the treatment of asthma is also presented in Table 6.
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Fig. 6. Adenosine receptor signalling pathways. Activation of the A₁ and A₃ adenosine receptors (ARs) inhibits adenylyl cyclase activity through activation of pertussis toxin-sensitive Gj proteins and results in increased activity of phospholipase C (PLC) via GPy subunits. Activation of the A₂A and A₂B ARs increases adenylyl cyclase activity through activation of Gs proteins. Activation of the A₂A AR to induce formation of inositol phosphates can occur under certain circumstances, possibly via the pertussis toxin-insensitive Ga15 and Ga16 proteins. A₂B AR-induced activation of PLC is through Gq proteins. All four subtypes of ARs can couple to mitogen-activated protein kinase (MAPK), giving them a role in cell growth, survival, death and differentiation. CREB, cAMP response element binding protein; DAG, diacylglycerol; IP₃, inositol 1,4,5-trisphosphate; PI₃K, phosphatidylinositol 3-kinase; PIP₂, phosphatidylinositol-4,5-bisphosphate; PK, protein kinase; PLD, phospholipase D; NF-κB, nuclear factor-κB.

Table 5. Characteristics and pharmacology of adenosine receptors.

<table>
<thead>
<tr>
<th>A₁AR</th>
<th>A₂A AR</th>
<th>A₂B AR</th>
<th>A₃AR</th>
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<tr>
<td>Agonists</td>
<td>CPA, CCPA, CHA, CGS 21680, S-ENBA</td>
<td>ATL146e, BAY 60-6583</td>
<td>IB-MECA (CF101)</td>
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<td>CV-1808, CVT-3146, MRE0740, MRE0094</td>
<td>2-CI-IB-MECA</td>
<td>MRS3558</td>
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</tbody>
</table>

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Antagonists: DPCPX, FSCPX, ZM 241385, KW IPDX, N-0861, BG-9719, BG-9929, WRC-0571, KW 3902, L-97-1, SLV320, EPI-2010

Transduction mechanisms: G
\text{iv} \downarrow \text{cAMP}; \uparrow \text{K}^+; G
\text{iv} \uparrow \text{cAMP}; G
\text{iv} \downarrow \text{cAMP}; \uparrow \text{Ca}^{2+} \text{channels}, \text{PLA}_2; G_{q/6} \text{NF-kB}, \text{PLC, PKC, IP3/DAG}

Table 6. Comparison of different potential therapeutic approaches targeting adenosine receptors in asthma$^{122}$. 

<table>
<thead>
<tr>
<th>A_1 AR antagonists</th>
<th>A_2A AR agonists</th>
<th>A_2B AR antagonists</th>
<th>A_3 AR agonists</th>
<th>A_3 AR antagonists</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potential effects</td>
<td>Bronchodilation and inhibition of Bronchoconstriction, inflammation, and airway remodeling</td>
<td>Inhibition of in inflammation</td>
<td>Inhibit Bronchoconstriction, inflammation, mucus hyperplasia</td>
<td></td>
</tr>
<tr>
<td>Disadvantages</td>
<td>CV side effects; tachyphylaxis; immune suppression</td>
<td>Reduce airway hydration; bronchoconstriction inflammation</td>
<td>Tachyphylaxis, immune suppression</td>
<td>Inflammation</td>
</tr>
<tr>
<td>Latest developments in asthma</td>
<td>GW328267X (Phase II; discontinued)</td>
<td>CVT 6883 (Phase I); QAF 805 (Phase Ib)</td>
<td>QAF 805 (Phase Ib)</td>
<td></td>
</tr>
<tr>
<td>Pharmaceutical company involved in AR drug discovery</td>
<td>Epigenesis Pharmaceuticals; Glaxo Group Ltd; Pfizer; Novartis</td>
<td>CV Therapeutics; Novartis</td>
<td>Can-File Biopharma</td>
<td>Novartis</td>
</tr>
</tbody>
</table>

AR, Adenosine receptor; CV, cardiovascular; ICSs, inhaled corticosteroids
Most promising prospects exist for treatment of arrhythmias, ischaemia of the heart and brain, pain, neurodegenerative diseases, sleep disorders, inflammation, diabetes, renal failure, cancer and glaucoma, and in cardiovascular imaging. High and intermediate levels of $\text{A}_1$ adenosine receptor (AR) expression were found in the brain, heart, adipose tissue, stomach, vas deferens, testis, spleen, kidney, aorta, liver, eye and bladder\textsuperscript{140}. The $\text{A}_{2A}$ AR is highly expressed in the striatum, nucleus accumbens and olfactory tubercle\textsuperscript{140}. High and intermediate expression levels were also found in immune cells, heart, lung and blood vessels. The $\text{A}_{2B}$ AR was generally expressed at low levels in almost all tissues\textsuperscript{140}. Rat testis has particularly high concentrations of $\text{A}_3$ AR mRNA, with moderate levels in lung. The highest levels of human $\text{A}_3$ AR mRNA have been found in lung and liver. $\text{A}_3$ ARs have been detected in various tissues including testis, lung, kidney, placenta, heart, brain, spleen, liver, uterus, bladder, jejunum, aorta, proximal colon and eyes.
Fig. 8. Adenosine receptors and pathophysiology of asthma. By acting on adenosine receptors (ARs), A1, A2A, A2B, and A3 ARs, adenosine released under conditions of cellular stress as seen in asthmatic airways produces bronchoconstriction and inflammation. The net effect of adenosine on ARs will depend on the relative expression of these receptors on different cell types in asthmatic airways, and is concentration-dependent, as adenosine frequently exhibits opposing effects through the activation of AR subtypes expressed on the same cells coupled to different G proteins and signaling pathways. By acting on A1 ARs on bronchial smooth muscle cells and afferent sensory airway nerves, adenosine produces bronchoconstriction. By acting on A1 ARs on inflammatory leukocytes such as neutrophils, monocytes, macrophages, and lymphocytes, adenosine produces proinflammatory effects. Activation of A2A ARs on the inflammatory cells suppresses the release of proinflammatory cytokines and mediators. Activation of A2A ARs coupled to Gs and adenylate cyclase may also lead to bronchial smooth muscle relaxation via the cAMP-PKA (cyclic adenosine monophosphate–protein kinase A) pathway. Activation of A2B ARs coupled to Gs and adenylate cyclase induce cytokine release from human bronchial epithelial and smooth muscle cells. Activation of A3 ARs on murine bone marrow-derived mast cells (BMMCs) regulates the release of cytokines. The effect of adenosine on A3 ARs is species dependent. In mice, rats, and guinea pigs, activation of A3 ARs by adenosine produces bronchoconstriction, airway inflammation, mast cell degranulation, and mucus hyperplasia. In humans, activation of A3 ARs by adenosine produces anti-inflammatory effects, inhibition of chemotaxis and degranulation of eosinophils and cytokine release from monocytes. Circled times denote inhibition.
Search of New Antagonist Ligands for A3 Adenosine Receptors from Molecular Modeling Point of View

The A3 AR is the only AR whose existence was not defined by employing pharmacological evidence because it was discovered by cloning151. In fact, this AR has been cloned from a variety of species including rat and human and it has been noted that species differences for the A3 receptor are larger than for other AR subtypes152.

Significant differences in sequence homology (72%) for A3 receptors have been observed between species153. The amino acid sequence of the human A3 AR is 49.5%, 43.2% and 39.9% identical in sequence to human A1, A2A and A2B AR respectively. Among the various species, rat A3 AR is significantly different from human (73.8% of identical sequence), whereas sheep A3 AR is closely related to the human receptor (85.2% of identical sequence)154. The rat A3 AR in particular behaves anomalously in ligand binding assays with different affinities for same ligands (antagonists) versus human A3 AR. For this reason, the hypothesis of the existence of two different A3 AR subtypes has been proposed but the question is yet to be answered155. Furthermore, tissue distribution of A3 AR is very different in rat as compared to man. In rat, A3 AR is expressed in high density in testis and mast cells and lower density in most other tissues. In human, highest A3 receptor densities are found in lungs, liver, and cells of the immune system. Lower levels have been detected in many other human tissues, including brain, heart and testes152.

The growing understanding of the physiological effects mediated by the A3 subtype, such as modulation of cerebral and cardiac ischemia64,143-145,154, inflammation, or normal and tumor cell regulation146-149, makes this receptor subtype an interesting target for various therapeutic interventions. In particular, highly selective A3 AR antagonists are being investigated as potential antiasthmatic152, anti-inflammatory, antiglaucoma, and cerebroprotective agents and recently, they have been described as potential therapeutics in the treatment of glioblastoma multiforme, colon cancer, and renal injury64. The Anti- and proinflammatory effects mediated by activation of A3 AR is presented in Fig. 9.
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Fig. 9. Anti- and proinflammatory effects mediated by activation of A3 AR. In rat mast cells, activation of A3 AR stimulates degranulation. In eosinophils, it inhibits chemotaxis, degranulation and superoxide anion generation ex vivo but not in the intact animal. In monocytes and macrophages, it inhibits TNF-α release through NF-κB pathway. In human neutrophils, it stimulates chemotaxis and inhibits superoxide anion generation.

During the past two decades, intensive efforts have been made to identify selective ligands for these receptor subtypes in order to facilitate pharmacological studies in vitro and in vivo. But a few such compounds are currently undergoing clinical trials for the treatment of several diseases; nevertheless, many of them have been discarded. The main problems include side effects due to the wide distribution of ARs, low water solubility, or lack of effects—in some cases perhaps due to low receptor density in the targeted tissue. In addition, the design, development, and commercialization of a drug is a tedious, time consuming and cost-intensive process.

For these reasons, any tool or technique that increases the efficiency of any stage of the drug discovery process is highly desirable. Computer-aided drug design
based on Quantitative Structure Activity Relationship (QSAR) methods is one such tool that can be used to increase the efficiency of the drug discovery process\textsuperscript{157,158}. The main paradigm of medicinal chemistry is that the biological activity, as well as physical, physicochemical, and chemical properties, of organic compounds depends on their molecular structure\textsuperscript{159}.

Based on this paradigm Crum-Brown and Fraser\textsuperscript{160} published the first QSAR in 1968. In spite of the great advances made in the field of theoretical drug design, even today this paradigm is guiding the discovery of new lead compounds. The main objective of this approach is to discriminate potent candidate molecules from inactive or less potent molecules\textsuperscript{161}. In order to obtain the structure–activity relationships on which these theoretical and computational methods are based, it is necessary to find appropriate representations of the molecular structures of drug compounds or the receptor itself. These representations are obtained through the so-called “molecular descriptors” in the case of the traditional QSAR or the protein target in the case of receptor modeling (ligand docking). The term “molecular descriptor” is used to characterize a specific aspect of a molecule. That is, they are numbers that contain structural information derived from the structural representation used for the molecules under investigation or unambiguously include measurable physical–chemical properties, and in the case of docking, the protein sequence and its spatial representation.

In the design of new biologically active compounds with docking techniques, homology modeling is of current interest, particularly in the case of AR antagonists. To perform a docking screen, the first requirement is the structure of a protein of interest. Usually, the structure is determined in the laboratory using a biophysical technique such as X-ray crystallography, or less often, NMR spectroscopy. This protein structure and a database of potential ligands serve as inputs to a docking program. This methodology has provided useful insights into ligand–receptor interactions. Several research groups have developed different methodologies to carry out a rhodopsin-based approach for the design of new adenosine ligand antagonists and have obtained excellent results\textsuperscript{162–168}.

On the other hand, QSAR studies can reduce the costly failures of drug candidates in clinical trials by filtering the combinatorial libraries. Virtual filtering can
eliminate compounds with predicted toxic of poor pharmacokinetic properties\(^{169,170}\) early in the process. It also allows for narrowing the library to drug-like or lead-like compounds\(^{171}\) and eliminating the frequent-hitters, that is, compounds that show unspecific activity in several assays and rarely result in leads\(^{172}\). Interpretation of created models gives insight into the chemical space in proximity of the hit compound. Later in the drug discovery process, accurate QSAR models constructed on the basis of the lead series can assist in optimizing the lead\(^{173,174}\). All these advantages can be very useful in the discovery of new potent adenosine antagonists with better pharmacological profile.

An interesting study on QSAR of A\(_3\) AR antagonists was carried out by Kunal Roy. Initially Roy\(^{175}\) worked with a set of 1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-one derivatives (Fig. 10A), reported by Colotta et al.\(^{176}\) and their affinity values were expressed as pK\(_a\) generated for displacement of specific \([^{125}\text{I}]\text{AB-MECA}\) binding at human A\(_3\) ARs expressed in HEK-293 cells\(^{177}\). The best models (Eq. 1) for this AR subtype are shown below:

\[
pK_a = -37.004 + 1.554f_{C-O} + 1.297q_{1.2} - 70.383q_{1.4}
\]

\(N = 27, \quad R = 0.911, \quad F = 37.200, \quad S = 0.280, \quad AVRES = 0.217\)

On the basis of the model, authors explain that the benzene moiety of the quinoxaline nucleus (evidence from the importance of the atoms C-1 and C-2) and the oxo group (atom O-14) on the triazole ring are essential for A\(_3\) binding affinity. The presence of alkyl substituents on the N-7 atom of the quinoxaline ring reduces the activity while the presence of an acylamino substituent at R1 increases the activity.

Based on the above analysis, the authors identify in the general structure of the compounds certain fragments that may provide the best probability of interaction with this specific AR subtype. The analysis is presented in (Fig. 10B).
Fig. 10. (A) General structure of 1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-one derivatives (B) Fragments of the general structure used in the study and their important interaction sites at A3 receptor.

The same author reported a second QSAR study\textsuperscript{178} with a set of 2-arylpyrazolo[3,4-c]quinoline derivatives (Fig. 11) with known A\textsubscript{3} receptor binding affinities\textsuperscript{179} expressed as pK\textsubscript{i}, generated for displacement of specific [\textsuperscript{125}I]AB-MECA binding at human A\textsubscript{3} ARs expressed in CHO cells\textsuperscript{177}. This research was modeled with Wang–Ford charges of different common atoms along with some indicator parameters and physicochemical variables in order to explore the selective pattern for the AR binding affinity in terms of structural requirements. The best models obtained and the statistical parameters for A\textsubscript{3} AR subtype are shown below:

\begin{align*}
\text{pk} & = 10.507 \quad \text{2.065_\text{DF}} \quad 27.374_\text{DF} \quad 0.000_\text{DF} \quad 0.433_\text{DF} \quad 1.433_\text{DF} \quad 0.130_\text{DF} \\
N & = 54, \quad R = 0.902, \quad F = 19.8, \quad S = 0.377, \quad \text{AVRES} = 0.262, \quad q^2 = 0.688 \\
\text{PRESS} & = 6.399, \quad \text{SDFP} = 0.434, \quad \text{PRESS} = 0.487, \quad \text{PRESS} = 0.336 \\
\text{pk} & = 1.754 \quad 0.623_\text{DF} \quad 1.330_\text{DF} \quad 0.756_\text{DF} \quad 0.543_\text{DF} \quad 0.880_\text{DF} \quad 0.193_\text{DF} \\
N & = 54, \quad R = 0.874, \quad F = 14.6, \quad S = 0.423, \quad \text{AVRES} = 0.296, \quad q^2 = 0.645 \\
\text{PRESS} & = 7.480, \quad \text{SDFP} = 0.469, \quad \text{PRESS} = 0.527, \quad \text{PRESS} = 0.375
\end{align*}
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\[ pk = 10.0 \pm 8 \quad 2.07 \pm 3 \quad 2^{-0.93}N_{2,3} - 0.75S_{12} \quad 1.34I_{\text{flat}} + 0.45MR \]  

were \( I'_{\text{CO}} \) and \( I'_{\text{pent}} \) indicate the presence or absence of a nuclear or extranuclear ketone (\( >\text{CO} \)) and of a cyclopentylamino substituent at the 4-position of the pyrazolo[3,4-c]quinoline nucleus, respectively; \( I_R \) represents the presence or absence of meta- or para-substituents larger than a hydrogen on the appended 2-phenyl moiety; \( I_{2\text{Me}} \) signifies the presence or absence of a 2-methyl group on the appended 2-phenyl moiety and MR is the molar refractivity.

According to the interpretation of these models the author reported that increase in negative charge on atom 7 (quinoline N) increases \( \text{A}_3 \) receptors binding affinity, while increase in negative charge on atom 13 (pyrazole N) decreases binding affinity. Further, the presence of a nuclear or extranuclear carbonyl group at the 4-position of pyrazolo[3,4-c]quinoline nucleus and of a meta or para substituent with higher bulk (size) than H on the appended 2-phenyl ring contribute to the \( \text{A}_3 \) binding affinity while the presence of an ortho-methyl group on the appended 2-phenyl ring decreases activity. Again, a 4-cyclopentylamino substituent increases \( \text{A}_3 \) binding affinity. Molar refractivity of the molecules also shows positive impact on the binding affinity.

The latest paper in this series described similar methodology and procedures\(^{180}\). In this QSAR study, a data set of 1,2,4-triazolo[5,1-]purine derivatives\(^{181}\) (Fig. 12) was considered. Their affinity values were expressed as \( \mu \text{C} \), generated for displacement of specific \(^{125}\text{I}\)AB-MECA binding at human \( \text{A}_3 \) ARs expressed in HEK-293 cells\(^{182}\), and lipophilicity, quantum chemical, and indicator parameters were used as molecular descriptors to describe \( \text{A}_3 \) affinity requirements for ARs.

\[ \text{Fig. 12. General structure of 1,2,4-triazolo[5,1-]purine derivatives.} \]
The best models obtained and their relevant statistical parameters for A3 AR subtype are shown below:

\[
pC = -16.562 + 2.174 \log P - 0.263 \log P^2 - 14.058q_{2-4} - 3.396l_{aro} \quad (5)
\]

N = 29, \quad R = 0.935, \quad F = 41.5, \quad S = 0.342, \quad AVRES = 0.255, \quad Q^2 = 0.796

PRESS = 4.500, \quad SDEP = 0.396, \quad SPRESS = 0.435, \quad Prep = 0.328

where pC = -log (IC\textsubscript{50}), l\textsubscript{aro} is an indicator variable that has a value of 1 if the atom C-3 is not directly connected to an aromatic nucleus and a value of 0 otherwise; q\textsubscript{2-4} represents the difference between the charges of its respective atoms in the molecule and the log P is the partition coefficient of octanol/water.

The negative coefficient of l\textsubscript{aro} variable suggests that an aromatic substituent conjugated with the triazole nucleus should be present at the R\textsubscript{2} position for improved A3 binding affinity. Once again, high negative charges on N-2 and N-4 provide binding affinity, as evidenced by the negative coefficient of q\textsubscript{2-4}. Finally, the difference of charge between N-2 and N-11 atoms (negative charge on the former should be higher and such a charge on the latter should be lower) together with the negative charge on N-7 provide A3 affinity and selectivity.

Recently, work by the same research group has been focused on the design of antagonists for the A3 AR. Bhattacharya et al\textsuperscript{183} developed a QSAR model with a data set of 1,2,4-triazolo[4,3-a]quinoxalin-1-one and 1,2,4-triazolo[4,3-a]quinoxaline-1,4-dione derivatives\textsuperscript{184} (Fig. 13); their affinity values were expressed as pK\textsubscript{i}, generated for displacement of specific \textsuperscript{125}IAB-MECA binding at human A3 ARs expressed in CHO cells\textsuperscript{177} and quantum chemical indices, lipophilicity (log P), physicochemical substituent constants (π, MR, α\textsubscript{p}) of phenyl ring substituents, and appropriate indicator parameters were used as predictor variables.

**Fig. 13.** General structure of 1,2,4-triazolo[4,3-a]quinoxalin-1-one and 1,2,4-triazolo[4,3-a]quinoxaline-1,4-dione derivatives.
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The statistical qualities of the multilinear regression equations were judged by their parameters, such as explained variance ($R_a^2$), correlation coefficient ($R$), standard error of estimate (S), and variance ratio (F), at specified degrees of freedom. The best model obtained according to these criteria and their relevant statistical parameters are shown below:

\[ pk_i = 4.654 + 1.913\sigma_p + 0.684I_{NO_2} \]  \hspace{1cm} (6)

\[ N = 17, \quad R = 0.872, \quad F = 22.2, \quad S = 0.465, \quad q^2 = 0.653 \]

\[ \text{PRESS} = 4.400, \quad \text{SDEP} = 0.507, \quad S_{\text{PRESS}} = 0.559 \]

The two models shown below were obtained by Bhattacharya et al.

\[ pk_i = 4.294 + 0.320f_3 + 0.518f_4 + 0.332f_5 - 0.396f_6 - 0.187f_7 \]  \hspace{1cm} (7)

\[ N = 17, \quad R = 0.923, \quad F = 12.7, \quad S = 0.411, \quad q^2 = 0.631 \]

\[ \text{PRESS} = 4.600, \quad \text{SDEP} = 0.524, \quad S_{\text{PRESS}} = 0.651 \]

\[ pk_i = 4.452 + 1.847\sigma_p + 0.792I_{NO_2} - 2.375q_{15} + 2.507q_{19} \]  \hspace{1cm} (8)

\[ N = 17, \quad R = 0.899, \quad q^2 = 0.648 \]

\[ \text{PRESS} = 4.400, \quad \text{SDEP} = 0.511, \quad S_{\text{PRESS}} = 0.608 \]

The study suggests the importance of R and R₁ substituents for A₃ binding affinity. The binding affinity increases with the presence of electron-withdrawing substituents at the R position. Furthermore, the charges on atoms C-15 and C-19 also play an important role in the determination of binding affinity. The presence of a nitro group as the R₁ substituent is also favorable for binding affinity.

Nevertheless, some of these conclusions were made from the interpretation of Equations (7) and (8), which are somewhat overfitted\(^{185,186}\).
Equations (7) and (8) break the rule of $p$ that was explained above taking values less than 4 (Eq. 21, $p = 2.84$; Eq. 22, $p = 3.4$), and this may indicate potential overfitting. Another indicator for determining a possible overfitting is $q^2$. The regression models obtained were validated by calculating $q^2$ values. The $q^2$ resulted from a “leave-one-out” (LOO) test, also known as cross-validation. In this process, a data point is removed from the set and the regression is recalculated; the predicted value for that point is then compared to its actual value. This procedure is repeated until each datum has been omitted once; the sum of squares of these deletion residuals can then be used to calculate $q^2$, an equivalent statistic to $R^2$. The $q^2$ values can be considered to be a measure of the predictive power of a regression equation, whereas $R^2$ can always be increased artificially by adding more parameters (descriptors). $q^2$ decreases if a model is overparameterized (to compare these values in Eqs. 6, 7, and 8), and is therefore a more meaningful summary statistic for QSAR models. Finally, another of these criteria was formulated by Akaike in 1973. Akaike’s information criterion (AIC) takes into account the statistical goodness of fit and the number of parameters that have to be estimated to achieve that degree of fit. This criterion is calculated using the following equation:

$$AIC = RSS \frac{(n + p')}{(n - p')^2}$$ (9)

where RSS is the sum of the squared differences between the observed ($y$) and estimated response ($y'$), $n$ is the number of compounds in the training set and $p'$ is the number of adjustable parameters in the model. When comparing models, the model that produces the minimum AIC value should be considered as potentially the most useful. In addition, the Kubinyi function (FIT) (Eq. 10) is closely related to the $F$ value. FIT is a useful parameter to assess the quality of models.

$$FIT = \frac{R^2(n - k - 1)}{(n + k^2)(1 - R^2)}$$ (10)

In the FIT expression $n$ is the number of compounds in the training set, $k$ the number of variables in the equation that describes the model, and $R^2$ is the squared correlation coefficient. The FIT criterion has a low sensitivity towards changes in $k$. 

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values, provided that they are small numbers, and a substantially greater sensitivity for large \( k \) values\(^{190}\). The best model will present the highest value for this function. The applications of these last two parameters (AIC and FIT) have been demonstrated in some publications\(^{191}\). According to calculated FIT and AIC values (Table 7) the variables included in Equations (7) and (8) do not support the quality of their statistical parameters.

**Table 7.** Values of AIC and FIT calculated for equations (6-8).

<table>
<thead>
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<th>Equations</th>
<th>AIC</th>
<th>FIT</th>
</tr>
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<tbody>
<tr>
<td>6</td>
<td>0.306</td>
<td>2.111</td>
</tr>
<tr>
<td>7</td>
<td>0.352</td>
<td>1.520</td>
</tr>
<tr>
<td>8</td>
<td>0.367</td>
<td>1.530</td>
</tr>
</tbody>
</table>

For this reason, it is estimated that these models are overfitting. Over the years, much discussion has centered on the problem of overfitting in QSAR models; a clear example published recently shows that models that include unnecessary predictors lead to worse decisions\(^{186}\). In drug discovery, for example, a mistaken decision to add irrelevant predictors can make predictions worse because the coefficients fitted to them add random variation to the subsequent predictions\(^{186}\). In addition, this experimental measurement has a relatively high experimental error and therefore the models in which there are too many fittings from a statistics point of view run the risk of overfitting of the model.

On the other hand, Bhattacharya et al. reported another QSAR study\(^{192}\) that involved the use of the same statistical techniques, descriptors and a data set of 30 thiazole and thiazole derivatives that are \( A_3 \) AR antagonists, previously published by Jung et al.\(^{193}\) (Fig. 14) and their affinity values were expressed as pKi, generated for displacement of specific [\(^{125}\)I]AB-MECA binding at human \( A_3 \) ARs expressed in CHO cells\(^{177,182}\).
Four QSAR models were obtained (Eqs. 11-14) and the authors found that the $A_3$ binding affinity increases with decreasing lipophilicity of the compounds and with the presence of a methyl or ethyl substituent at C-13. The binding affinity was also found to decrease when a tert-butyloxy group is present at the same position. In addition, the phenyl ring substituents should be such that they increase the negative charges on C-2 and C-5, while the R substituent at C-13 should not be more hydrophobic than methyl or ethyl. Furthermore, the thiadiazole nucleus is preferred over the thiazole nucleus for the binding.

\[
pK_i = 4.030 - 3.951(\pm 2.415)q_2 - 2.623(\pm 2.136)q_3 + 2.441(\pm 1.489)q_7 \\
- 0.275(\pm 0.261) \log P - 1.498(\pm 0.763)I_{OBU-1} + 0.895(\pm 0.614)I_{Me-\text{Et}}
\]  
\[N = 30, \quad R^2 = 0.744, \quad R^2 = 0.797, \quad F(df6, 23) = 15.0, \quad s = 0.483\]

\[q^2 = 0.689, \quad SDEP = 0.523, \quad S_{PRES} = 0.597, \quad PRESS = 8.2\]

\[
pK_i = 4.401 + 0.280(\pm 0.118)f_2 + 0.415(\pm 0.118)f_3 - 0.368(\pm 0.118)f_4 \\
- 0.239(\pm 0.118)f_6 - 0.424(\pm 0.118)f_7 + 0.459(\pm 0.118)f_8
\]  
\[N = 30, \quad R^2 = 0.895, \quad R^2 = 0.916, \quad F(df6, 23) = 42.0, \quad s = 0.310\]

\[q^2 = 0.870, \quad SDEP = 0.338, \quad S_{PRES} = 0.386, \quad PRESS = 3.4\]
where $q_x$ were the Wang-Ford charges of different atoms (x) of the analogues; $I_{\text{Bu-t}}$ is an indicator variable having a value 1 if tert-butyloxy group is present at R position, value 0 otherwise; $I_{\text{Me-Et}}$ is an indicator variable having value 1 if $R = \text{methyl}$ or ethyl, value 0 otherwise and $f_x$ represent factors loadings and have the character of correlation coefficients between the common factors and the variables.

Another QSAR study for A3 ARs was reported by Borghini et al.\(^{194}\), who used a data set of thiazole and thiadiazole antagonist analogues (very similar to those studied by Bhattacharya et al.\(^{192}\)) reported in the literature\(^{193}\) and their affinity values were expressed as $pK_i$, generated for displacement of specific $[^{125}\text{I}]\text{AB-MECA}$ binding at human A3 ARs expressed in CHO cells. The use of CODESSA software led to QSAR equations (15) based on four descriptors for the adenosine A3 receptor ligands with good statistical parameters.

\[
pK_i = 3.083 - 1.940(\pm 2.072)q_1 - 11.413(\pm 3.878)q_8 - 13.611(\pm 5.005)q_9 \\
- 0.038(\pm 0.029)(\log P)^2 - 1.556(\pm 0.722)I_{\text{Bu-t}} \tag{13}
\]

\[
pK_i = 3.686 - 1.932(\pm 2.150)q_2 - 11.413(\pm 4.012)q_8 - 13.741(\pm 5.189)q_9 \\
- 0.311(\pm 0.260)\log P - 1.518(\pm 0.747)I_{\text{Bu-t}} \tag{14}
\]

where $q_x$ were the Wang-Ford charges of different atoms (x) of the analogues; $I_{\text{Bu-t}}$ is an indicator variable having a value 1 if tert-butyloxy group is present at R position, value 0 otherwise; $I_{\text{Me-Et}}$ is an indicator variable having value 1 if $R = \text{methyl}$ or ethyl, value 0 otherwise and $f_x$ represent factors loadings and have the character of correlation coefficients between the common factors and the variables.

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\[
pK_i = -6.17(\pm 2.47) + 17296(\pm 260)\frac{\text{HACA}_2}{\text{TMSA}} + 22.73(\pm 4.44)\text{RNH} \\
+ 46.56(\pm 10.83)\text{MPCS} - 0.382(\pm 0.00935)\text{WNSA}_3 \tag{15}
\]

\[
N = 30, \quad R^2 = 0.775, \quad R^2 = 0.814, \quad F(5, 24) = 21.0, \quad s = 0.452
\]

\[
q^2 = 0.753, \quad SDEP = 0.466, \quad S_PRES = 0.521, \quad PRESS = 6.5
\]

\[
pK_i = 3.686 - 1.932(\pm 2.150)q_2 - 11.413(\pm 4.012)q_8 - 13.741(\pm 5.189)q_9 \\
- 0.311(\pm 0.260)\log P - 1.518(\pm 0.747)I_{\text{Bu-t}} \tag{14}
\]

\[
N = 30, \quad R^2 = 0.762, \quad R^2 = 0.803, \quad F(5, 24) = 19.6, \quad s = 0.465
\]

\[
q^2 = 0.739, \quad SDEP = 0.479, \quad S_PRES = 0.536, \quad PRESS = 6.9
\]

where $q_x$ were the Wang-Ford charges of different atoms (x) of the analogues; $I_{\text{Bu-t}}$ is an indicator variable having a value 1 if tert-butyloxy group is present at R position, value 0 otherwise; $I_{\text{Me-Et}}$ is an indicator variable having value 1 if $R = \text{methyl}$ or ethyl, value 0 otherwise and $f_x$ represent factors loadings and have the character of correlation coefficients between the common factors and the variables.

Another QSAR study for A3 ARs was reported by Borghini et al.\(^{194}\), who used a data set of thiazole and thiadiazole antagonist analogues (very similar to those studied by Bhattacharya et al.\(^{192}\)) reported in the literature\(^{193}\) and their affinity values were expressed as $pK_i$, generated for displacement of specific $[^{125}\text{I}]\text{AB-MECA}$ binding at human A3 ARs expressed in CHO cells. The use of CODESSA software led to QSAR equations (15) based on four descriptors for the adenosine A3 receptor ligands with good statistical parameters.

\[
pK_i = -6.17(\pm 2.47) + 17296(\pm 260)\frac{\text{HACA}_2}{\text{TMSA}} + 22.73(\pm 4.44)\text{RNH} \\
+ 46.56(\pm 10.83)\text{MPCS} - 0.382(\pm 0.00935)\text{WNSA}_3 \tag{15}
\]

\[
N = 26, \quad R^2 = 0.700, \quad F = 12.23, \quad s = 0.559, \quad q^2 = 0.548
\]

\[
R^2_{\text{EXT}} = 0.676, \quad k = 1.061, \quad R^2_0 = 0.672, \quad \frac{R^2-R^2_0}{R^2} = 5.33 \times 10^{-3}
\]
where HACA _ 2 means total charge weighted HACA, HACA means hydrogen-acceptor charged surface area, and TMSA means total molecular surface area; RNH is the constitutional descriptor relative number of H atoms; MPCS is the minimum partial charge on a S atom; WNSA _ 3 is the total surface weighted PNSA and PNSA means partial negative surface area.

Moreover, the results appear to furnish useful suggestions for the design of new ligands to act as antagonists for the adenosine A3 receptors and confirm the results previously obtained by Bhattacharya et al. The model suggests the importance of small alkyl groups at the position R, since they increase the contribution coefficient of this descriptor RNH more than aromatic rings. It is only affected by the amide substituent since it shows the highest variability. Also the presence of a second N atom in the ring, near the S atom, probably affects its partial charge, because of its electronegativity. This suggests that thiadiazole should be preferred to thiazole derivatives.

According to the authors, the electrostatic descriptor WNSA_3 reflects both the negative charge and the total molecular surface properties. It is characterized by a negative sign in the QSAR equation and shows the importance of small molecules endowed with only a weak negative charge. The obtained results are in agreement with the structure-based studies on ligand–receptor interactions recently described by Jacobson et al.

In this respect, Li et al. using the same methodology, obtained a QSAR model for a set of pyridine derivatives (Fig. 15) with known A3 binding data and their affinity values were expressed as pKi, generated for displacement of specific [125I]AB-MECA binding at human A3 ARs expressed in HEK cells with the intention of identifying the structural features responsible for affinity.

Fig. 15. General structure of pyridine derivatives used by Li et al. The substituents at the R2, R3, R4, R5 positions were alkyl, alcohol and fluorinatedalkyl; the substituent at R6 was phenyl.
Several prediction and validation statistical parameters of the CoMFA model obtained are shown in Table 8.

**Table 8. Statistics for the calibration and validation of CoMFA model.**

<table>
<thead>
<tr>
<th>Number of compounds</th>
<th>A1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Principal components</td>
<td>41</td>
</tr>
<tr>
<td>R²</td>
<td>0.667</td>
</tr>
<tr>
<td>R²ᵤᵤ</td>
<td>0.968</td>
</tr>
<tr>
<td>Fᵩᵤᵤ</td>
<td>141.670</td>
</tr>
<tr>
<td>p value</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>R²ₘᵝ</td>
<td>0.931</td>
</tr>
<tr>
<td>SEP</td>
<td>0.185</td>
</tr>
<tr>
<td>std dev</td>
<td>0.022</td>
</tr>
</tbody>
</table>

The results show that the CoMFA-derived QSAR for pyridine derivatives exhibits a good crossvalidated correlation, indicating that it is highly predictive. The high bootstrapped $R^2$ value and the small standard deviation suggest a high degree of confidence in the analysis. In addition, analysis of the model suggests that around the C-2 position of the pyridine moiety the presence of bulky substituents enhances the affinity. The two ester groups at the 3- and 5-positions are also surrounded by green polyhedra, suggesting that the presence of alkyl substituents increases $A_3$ receptor affinity. Another important region of bulk tolerance was found around the meta position of the phenyl ring at the C-6 position of pyridine. In fact, a meta-chloro derivative displayed a favorable affinity. In contrast, the region of space around the C-4 position of the pyridine moiety was contained within a yellow polyhedron, suggesting that bulky substituents are not tolerated by the receptor at this position. Both styryl and phenylpropargyl derivatives were among the least active compounds. Substituents with high electron density were also accepted in the side chain of both ester groups at the 3- and 5-positions.

Moro et al.\(^{197}\) published an interesting prediction strategy for the design of new antagonists by combining target-based and ligand-based drug design approaches to
define a novel pharmacophore model for the human A₃ receptor. High throughput molecular docking and CoMFA analysis were used in tandem to assemble a new target-based pharmacophore model (Fig. 16). A total of 106 pyrazolotriazolopyrimidine derivatives (Fig. 17) were included in the training set used to generate the QSAR model for human A₃ receptors.

![Fig. 16. Scheme representing the combined target-based and ligand drugdesign approach proposed by Moro et al.](image)

Fig. 16. Scheme representing the combined target-based and ligand drugdesign approach proposed by Moro et al.

![Fig. 17. General structure of pyrazolotriazolopyrimidine derivatives used by Moro et al. The substituents at R and R₁ were mainly alkyl, aromatic and aromatic amide groups.](image)

Fig. 17. General structure of pyrazolotriazolopyrimidine derivatives used by Moro et al. The substituents at R and R₁ were mainly alkyl, aromatic and aromatic amide groups.

Binding of [³H]MRE3008-F20 to CHO cells transfected with the human recombinant A₃ ARs was performed according to Varani et al. and PLS techniques were carried out to find the best correlation. The model obtained with six principal components based on a least-squares fit gave a correlation with a cross-validated value
of 0.840. The noncross-validated PLS analysis was repeated with the optimum number of components, as determined by the cross-validated analysis, to give an $R^2$ of 0.922. In order to obtain statistical confidence limits, the noncross-validated analysis was repeated with 10 bootstrap groups, which yielded an $R^2$ of 0.802 and standard deviation of 0.022. A summary of these and other statistical results is shown in Table 9.

**Table 9. Statistics for the calibration and validation of CoMFA model.**

<table>
<thead>
<tr>
<th></th>
<th>$A_{3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of compounds</td>
<td>106</td>
</tr>
<tr>
<td>Principal components</td>
<td>6</td>
</tr>
<tr>
<td>$^aR^2_{cv}$</td>
<td>0.840</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.922</td>
</tr>
<tr>
<td>$^bF_{test}$</td>
<td>141.670</td>
</tr>
<tr>
<td>$p$ value</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>$^cR^2_{bs}$</td>
<td>0.802</td>
</tr>
<tr>
<td>SEP</td>
<td>0.185</td>
</tr>
<tr>
<td>$^d$ Std dev</td>
<td>0.022</td>
</tr>
</tbody>
</table>

*Standard error of prediction.
*Ratio of $R^2$-explained to $R^2$-unexplained.
*$R^2_{bs}$ is $R^2$ after bootstrapping.
*Standard deviation column belongs with the bootstrapping $R^2$.

The CoMFA-derived QSAR for pyrazolotriazolopyrimidine derivatives exhibited a good crossvalidated correlation, indicating that it was highly predictive. Cross-validation provides information concerning the predictive ability of the QSAR data set by minimizing the occurrence of chance correlations in the QSAR model. The high bootstrapped $R^2$ value and small standard deviation suggest a high degree of confidence in the analysis. In addition, the authors included a test set of 17 new derivatives predicted with these approximations reporting good results ($R^2 = 0.873$). Finally, the analysis showed that all steric and electrostatic features are coherent with the structure–activity relationship already described during the molecular docking analysis. In particular, the steric bulk favorable region is found near the N$^5$-carbamoyl moiety. The green polyhedron fits nicely with the receptor region around the phenyl ring of the carbamoyl moiety, characterized by the three nonpolar amino acids Ile-98, Ile-186, and Leu-244. Moreover, from an electrostatic point of view the regions around the
N³-substituents and the N³-carbamoyl moiety are complementary regarding Ser-175, His-95, Ser-247, and His-272.

A continuation of this work was reported by the authors, who used autocorrelation of molecular electrostatic potential (MEP) surface properties combined with PLS analysis as an alternative to generate ligand-based 3D-QSAR. In this research, two alternative PLS models were derived—one starting from CoMFA descriptors and the other starting from the autocorrelation descriptors. The unique characteristic of this work is the introduction of autocorrelation vectors as molecular descriptors for the PLS analysis applied to the modeling of AR antagonists. The autocorrelation allows the comparison of molecules (and their properties) with different structures and with different spatial orientations without any previous alignment. In particular, MEP was the property computed and its information was encoded in autocorrelation vectors.

The 3D spatial distribution and the electrostatic potential values are in fact largely responsible for the binding of a substrate to its receptor binding site. Validation was performed with an external test set and the results of the model using the autocorrelation descriptors was $R^2 = 0.87$—higher than that obtained with the CoMFA methodology. The preliminary results seem to indicate that this new alternative approach could robustly compete with the already well-established CoMFA approach. In particular, it was suggested that this approach could be a very interesting tool to filter a large structural database in several virtual screening applications.

Another application of CoMFA was reported by Moro et al. In this investigation a total of 36 flavonoid derivatives were included in the training set used to generate the $A_3$ adenosine QSAR model. The binding affinity was expressed as $K_i$ values generated for displacement of specific $[^{125}]$IAB-MECA binding at human $A_3$ ARs expressed in HEK cells. These molecules were classified into six families depending on the chemical structure: flavonols, flavones, flavanones, dihydroflavanols, furylchromones, and naphthoflavones (Fig. 18). Validation of the resulting model was carried out with a test set consisting of three molecules.

This model belongs to the same work discussed above in the $A_1$ and $A_{2A}$ sections inconvenient, for the same reason with the validation process. Some
Introduction

relevant statistical parameters of this QSAR model to corroborate the predictive power of it model, as can be seen in Table 10.

Fig. 18. General structure of several flavonoid derivatives used by Moro et al. The substituents at different positions were alkyl, ether and phenyl.

Table 10. Statistics for the calibration and validation of CoMFA model.

<table>
<thead>
<tr>
<th></th>
<th>A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of compounds</td>
<td>36</td>
</tr>
<tr>
<td>Principal components</td>
<td>6</td>
</tr>
<tr>
<td>$^a R^2_{cv}$</td>
<td>0.583</td>
</tr>
<tr>
<td>$^b R^2$</td>
<td>0.957</td>
</tr>
<tr>
<td>$^c F_{test}$</td>
<td>119.530</td>
</tr>
<tr>
<td>p value</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>$^c R^2_{bs}$</td>
<td>0.974</td>
</tr>
<tr>
<td>SEP</td>
<td>0.108</td>
</tr>
<tr>
<td>$^d$ Std dev</td>
<td>0.012</td>
</tr>
</tbody>
</table>

$^a$ Standard error of prediction.
$^b$ Ratio of $R^2$-explained to $R^2$-unexplained.
$^c$ $R^2_{cv}$ after bootstrapping.
$^d$ Standard deviation column belongs with the bootstrapping $R^2$.

The developed 3D-QSAR model proposes that bulky substituents are not tolerated at the C-2 position of the chromone moiety. Bulky groups are also predicted to be tolerated around the C-6 position of the chromone moiety for $A_3$ receptor binding. In fact, the presence of a chloro substituent in this position is tolerated. Also the area of bulky tolerance surrounding the ortho position of the phenyl ring suggests that bulky
substituents at this position may increase A3 affinity. In addition, the para position of the phenyl ring indicates that substituents with higher electron density exert a negative effect on the affinity, in agreement with the experimental data that alkoxy substituents in this position decrease the A3 receptor affinity.

**Adenosine Receptors Selectivity Based on QSAR Models: The Most Important Limitations**

Literature reveals several QSAR studies for predicting the four AR subtype antagonist affinities but only a few such studies could address the selectivity issue. The present scenario demands the kind of analysis which could allow designing new more selective antagonists to a specific subtype in humans. This is a very controversial problem since there are big differences among the ARs in the various mammalian species under study.

One reason for ambiguous results may be species differences in ARs. Initial studies\(^{206,207}\) showed differences in the potency of various ligands at binding sites in rats, calves and guinea pigs' brains: the antagonist 1,3-diethyl-S-phenylxanthine showed considerable differences in affinity for brain A1 receptors. Other studies indicate that the \([^3H]xanthine amine congener of the antagonist 1,3-dipropylxanthine also shows dramatic species differences in affinity for brain A_1 receptors\(^{208}\). In connection to this, Ukena et al.\(^{209}\) provided strong evidence for well-defined species differences in the recognition site on brain A_1 AR antagonists. The differences are strongly dependent on specific molecular features such as the presence of an aryl or cycloalkyl substituent, which greatly enhances affinity at the calf brain A_1 receptor. Thus, some antagonists can be 200-fold more potent in calves than in guinea pigs brain. Regardless of the nature of the alterations in the brain A_1 receptor from different species, the results provide a caution against extrapolating profiles of antagonist potencies from one species to another. Also similar results were provided by Ferkany et al.\(^{201}\) confirming and extending the previous reports concerning interspecies differences in adenosine A_1 recognition sites in mammalian brain tissue. The authors concluded that the use of bovine tissue to evaluate new compounds at A_1 receptor would seem inappropriate if the compounds are targeted for therapeutic use in humans. Furthermore, the
distribution of the enzyme 50-nucleotidase, a regulator of adenosine availability, is species-dependent and Verma and Marangos found interspecies differences in adenosine uptake sites in brain. Finally, the effectiveness of selective phosphodiesterase inhibitors as positive inotropic agents also shows a species dependence. Literature shows that all the A1 AR QSAR studies reported have been developed using experimental data measurements in rat and bovine species.

This means that based on the previous analysis, the results of these models should not be extrapolated to humans. Therefore, new efforts are necessary for developing models using biological data in human A1 ARs. On the other hand, Stone et al. found that A2 AR antagonists in rabbit, calf, and human tissue were significantly different in terms of their activity for the high-affinity [3H]NECA binding site than in mouse, guinea pig, or rat. So far, seven studies have been developed, but only three of them have academic use because they do not take into account the actual division at the A2 ARs (A2A vs A2B). Besides the other four, only two have been obtained with experimental affinity values for human receptors. Therefore, additional efforts are required to develop QSAR models for this kind of human receptors. Nevertheless, the A3 receptor subtype is the most seriously studied one and all the QSAR models have been obtained with affinity data from human; this is the reason why it is highly favorable for obtaining new analogues with therapeutic applications. This receptor has important differences for different mammalian species to another like the other AR subtypes. Xiao-duo et al. reported that at A3 ARs a detailed examination of the sequence showed that there are six residues in the transmembrane region that are conserved between rat and human A3 receptors and they are absent in sheep receptors and the affinities of 8-aryl xanthines in the rat, rabbit, and gerbil brain A3 receptors were considerably less than the previously reported affinities at cloned sheep and human A3 receptors. For these reasons, the selectivity analysis based on the QSAR studies that have been developed up to now, are difficult to perform and probably without utility due to the species differences mentioned before.

However, research in this way showed some interesting results. One of these works has been conducted by Moro et al. where similarities were seen in the topology of steric and electrostatic regions with the A1 and A3 receptors but not the A2A receptor.
This is consistent with the structure–activity relationship data for which A1–A3 similarity has been demonstrated\(^{182}\). Also they found that the nature of C-2 chromone phenyl ring substituents was considered important for the binding affinity for all ARs. According to the authors, this phenyl ring may interact in a similar region of space inside the receptor binding site. An interesting consideration concerning A1/A3 selectivity was deduced from CoMFA contour map analysis. An important green region (favorable steric bulk interaction) was located around the 20-position of the phenyl ring in the A3 model. This is consistent with the experimental data for a few compounds, which were the most selective flavonoid compounds for human A3 receptors also the presence of a C-6 substituent in the chromone moiety is well tolerated and increases the A1/A3 selectivity. Nevertheless, this selectivity study has the difficulty that the affinity data used for the A1 and A2A receptors were from rats and the data for the A3 one from humans. Similar studies with the same problem were reported by Borghini et al.,\(^{194}\) Roy\(^{175}\) and Roy et al.\(^{178}\) Only one research developed so far takes into account these species differences\(^{180}\). The conclusions were somewhat general and once again more effort must be directed toward this area as the authors had very well said. However, this QSAR study helps to elucidate the physicochemical requirements of 1,2,4-triazolo[5,1-\(i\)]purine derivatives for selective binding with the A3 receptor over the A2A receptor.

**Progress in the Pursuit of Therapeutic A3 Adenosine Receptor Antagonists**

The study of the A3 adenosine receptor (A3 AR) represents a rapidly growing and intense area of research in the adenosine field. In particular, A3-selective AR antagonists have been postulated as novel anti-inflammatory, antiallergic and antiasthmatic agents; recent studies also indicated a possible employment of these derivatives as antitumor agents. In recent years many efforts have been made to search for potent and selective hA3 AR antagonists. A synopsis of A3 AR-regulated pathways and functions is provided in Fig. 19. It appears evident that a plethora of biological functions have been attributed to the A3 AR in ischemic and inflammatory pathologies, and substantial efforts in medicinal chemistry have been directed at developing agonists and antagonists that target this AR subtype\(^{215}\).
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Fig. 19. Synopsis of the A3 adenosine receptor (A3 AR) regulated pathways and functions. Activation of the A3 AR results in the modulation of different intracellular pathways. Classically, this adenosine receptor subtype is coupled to the inhibition of adenylyl cyclase, leading to a reduction in cAMP levels. In addition, it may activate phospholipase C (PLC), through Gβγ subunits, inducing an increase in intracellular calcium and activation of protein kinase C (PKC). Recently, it has also been demonstrated that it is coupled to mitogen-activated protein kinases (MAPKs), suggesting its involvement in cell growth, survival, death and differentiation. Activation of the A3 AR subtype induces protective effects in the CNS, heart and lung, and both pro- and anti-inflammatory effects in peripheral blood cells.

As a result, there are currently A3 AR agonists in clinical phases for several autoimmune diseases, such as RA. Unfortunately, there are no A3 AR antagonists currently in clinical development, but a number of molecules are in biological testing as therapeutic agents for asthma and COPD, glaucoma, and stroke, which are waiting to enter the clinical arena. This is only the starting point of more expensive and challenging work, and it is likely that, with the availability of both selective ligands and
animal models, several roles of the A3 AR that are currently ambiguous will be clearer in the near future. This will allow the chemistry and pharmacology of the A3 AR to be utilized clinically with the development of selective molecules for this important target that may improve the outcomes of patients with a number of diseases.

**Xanthine Derivatives**

Natural antagonists for ARs, such as caffeine and theophylline, show in general low affinity for the A3 AR subtype\(^{215}\). Different positions of the xanthine core have been modified with the aim of improving A3 AR affinity. A series of tricyclic imidazo[2,1-i]purinones and ring-enlarged analogues derived from xanthine derivatives has been prepared as AR antagonists. In comparison with xanthines, the tricyclic compounds exhibit increased water solubility due to a basic nitrogen atom, which can be protonated under physiological conditions\(^{216}\). Among this series PSB-10, 8(R)-ethyl-4-methyl-2-(2,3,5-trichlorophenyl)-4,5,7,8-tetrahydro-1H-imidazo[2,1-i]purin-5-one (Fig. 20), is a high-affinity ligand for A3 ARs (hA3, \(K_i = 0.43\) nM) with high selectivity over hA1 and hA2A ARs (\(K_i = 1700\) and 2700 nM, respectively). The compound showed inverse agonist activity in binding studies in CHO cells expressing recombinant hA3 ARs (IC50 = 4 nM)\(^{217}\).

Another similar compound is 2-(4-bromophenyl)-7,8-dihydro-4-propyl-1H-imidazo[2,1-i]purin-5(4H)-one, also named KF-26777 (Fig. 20), endowed with subnanomolar affinity to hA3 ARs (\(K_i = 0.20\) nM) and high selectivity over hA1, hA2A, and A2B ARs (9000-, 23 500-, and 31 000-fold, respectively). It concentration-dependently inhibited 2-chloro-N6-(3-iodobenzyl)-N-methyl-5'-carbamoyladenosine (CI-IB-MECA)-induced [\(^{35}\)S]guanosine 5'-O-(3-thiotriphosphate) ([\(^{35}\)S]-GTPyS) binding to human embryonic kidney 293 cells (HEK293) (IC50 = 270 nM) and enhanced intracellular Ca\(^{2+}\) concentration in human promyelocytic cells (\(K_i = 0.42\) nM). This agent was indicated as potential lead molecule for development for treatment of brain ischemia and inflammatory diseases such as asthma\(^{218}\).
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In this field of research the triazolopurine derivatives in which the xanthine structure is extended are also reported. One example is OT-7999 (Fig. 20), which proved to be a potent and selective hA3 AR ligand. In receptor binding assays, OT-7999 displayed high affinity for the A3 AR (Ki = 0.95 nM) and >10 500-fold selectivity relative to other AR subtypes. Significant reductions in intraocular pressure were obtained in cynomolgus monkeys at 2-4 h following topical application to the eye of OT-7999 (500 mcg).¹⁸¹,²¹⁹

1,4-Dihydropyridine and Pyridines

Starting from the experimental observations that 1,4-dihydropyridines bind A₁ adenosine receptors in the rat brain,²²⁰,²²¹ Jacobson et al. used the 1,4-dihydropyridine nucleus as a template for probing the SAR profile at the A3 AR subtype.²²² SAR studies of adenosine receptor antagonists indicated that sterically bulky groups are well tolerated at the 4-, 5-, and 6-positions. The combination of substitutions led to the discovery of MRS 1097 (2-methyl-6-phenyl-4-styryl-1,4-dihydro-pyridine-3,5-dicarboxylic acid diethyl ester, Fig. 21), MRS 1191, (2-methyl-6-phenyl-4-phenylethynyl-1,4-dihydro-pyridine-3,5-dicarboxylic acid 5-benzyl ester, Fig. 21), and MRS 1334 (2-methyl-6-phenyl-4-phenylethynyl-1,4-dihydro-pyridine-3,5-dicarboxylic acid 3-ethyl ester 5-(4-nitro-benzyl) ester, Fig. 21) as the first A₃ antagonists related to 1,4-dihydropyridines. In this study, they also synthesized pyridine derivatives through oxidation of the corresponding 1,4-dihydropyridine. In this class of compounds, small groups at the 4-position were found to be essential such as in MRS 1523 (6-ethyl-5-
ethylsulfanylcarbonyl-2-phenyl-4-propyl-nicotinic acid propyl ester, Fig. 21), which showed favorable affinity at the hA3 AR subtype. Comparing the structural requirements for the two related classes of compounds indicated that bulky substituents at the 4-position and a 5-benzyl ester, which are affinity enhancing in dihydropyridines, are not well tolerated in the pyridine series for A3 receptor binding. At other positions, structural parallels occur between corresponding dihydropyridine and pyridine analogues.

Fig. 21. 1,4-Dihydropyridine and pyridine derivatives as A3 AR antagonists.

Pyrazolo-triazolo-pyrimidines

The pyrazolo-triazolo-pyrimidine nucleus, due to its strong structural correlation with the nonselective antagonists CGS-15943, and the adenine nucleus present in the endogenous modulator adenosine (Fig. 22), has been strongly investigated in the past decade as a prototypical template for adenosine antagonists. The triazolo-quinazoline derivative CGS-15943 represented the starting point for searching for new potent and selective hA3 adenosine receptor antagonists. MRS-1220, a 5-N-phenylacetyl derivative of CGS-15943, in receptor binding studies displayed Kᵢ values of 305 ± 51, 52.0 ± 8.8, and 0.65 ± 0.25 nM for rat A₁, A₂A, and hA₃ receptors, respectively, being 470- and 80-fold selective for hA₃ ARs vs rat A₁ and A₂A ARs, respectively. MRS-1220 (Fig. 23) also antagonized the effects of an A₃ agonist in functional assays.
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CGS 15943 Adenosine

Fig. 22. CGS-15943 as A$_3$ AR antagonist.

An innovative series of tricyclic compounds (MRE series) reported by Baraldi’s group represented new selective A$_3$ AR antagonists. In this class attention was focused on the N$^6$ patterns of substitution due to the quite complete inactivity of the N$^7$-substituted derivatives at the hA$_3$ subtype (e.g., SCH-58261).

Fig. 23. Pyrazolo-triazolo-pyrimidines as A$_3$ AR antagonists.

MRE-3008-F20 (Fig. 23), one of several high affinity antagonists, is an A$_3$ AR ligand ($K_i = 0.29$ nM against 4-aminobenzyl-5'-N-methylcarboxamidoadenosine ([$^125$I]-AB-MECA) binding to human receptors expressed in HEK293 cells with high selectivity over rat A$_1$ and A$_2A$ ARs ($K_i > 10000$ and 1993 nM, respectively) as well as hA$_1$ and hA$_2A$ ARs ($K_i = 1197$ and 141 nM, respectively). The compound showed antagonist activity in a functional assay being capable of blocking the effect of IB-MECA on cAMP
production in CHO cells (IC\textsubscript{50} = 4.5 nM)\textsuperscript{228-230}. The tritium-labeled compound was able to bind hA\textsubscript{3} ARs expressed in CHO cells with a KD value of 0.82 nM and a B\textsubscript{max} value of 297 fmol/mg protein and represents the first high-affinity, selective radiolabeled antagonist for this subtype resulting in a useful tool for characterization of A\textsubscript{3} ARs in both normal and pathological conditions\textsuperscript{231}. The isosteric replacement of the phenyl with a 4-pyridyl moiety provided higher hydrosolubility and led to the first water-soluble hA\textsubscript{3} antagonist (MRE-3005-F20, Fig. 23) which is an ideal candidate for the pharmacological and clinical investigations of the hA\textsubscript{3} AR subtype\textsuperscript{166}.

In molecular modeling studies reported by Moro et al. on pyrazolo-triazolo-pyrimidines, a combined target-based and ligand-based drug design has been carried out to define a novel pharmacophore model for the hA\textsubscript{3} AR antagonists. A high-throughput docking strategy has been applied on the pyrazolo-triazolo-pyrimidine series. All low-energy docked conformations have been superimposed and used to characterize the common features crucial to the recognition process. A novel target-based pharmacophore model has been proposed for human A\textsubscript{3} AR antagonists. A CoMFA (comparative molecular field analysis) approach has been used as an alternative scoring function for prediction of ligand receptor binding affinity. The new target-based pharmacophore model was coherent with the structure-activity relationships collected on the pyrazolo-triazolo-pyrimidine analogues\textsuperscript{232,233}. Moreover, very recently Botta, Martinelli, and Baraldi et al. performed a pharmacophoric study using the software Catalyst, which yielded three different common feature hypotheses for antagonists of the hA\textsubscript{3} AR. The three pharmacophores referred to a recurring scheme consisting of three hydrophobic interactions lying at the vertexes of a triangle. They seemed particularly good in handling pyrazolo-triazolopyrimidine derivatives\textsuperscript{234}. These results confirm the importance of this tricycle as the most potent class of A\textsubscript{3} AR antagonists.

Isouquinoline and Quinazoline Urea Analogues as Antagonists for the Human Adenosine A\textsubscript{3} Receptor

A structure-affinity analysis reported by IJzerman et al.\textsuperscript{235} indicated that on the 2-position of the quinazoline ring or the equivalent 3-position of the isoquinoline ring a
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phenyl or heteroaryl substituent increased the A3 AR affinity in comparison to unsubstituted or aliphatic derivatives. Combination of the optimal substituents in the two series led to the potent hA3 AR antagonist N-(2-methoxyphenyl)-N'-(2-(3-pyridyl)quinazolin-4-yl)urea (VUF5574, Fig. 24) with a $K_i$ value of 4 nM and a selectivity of at least 2500-fold vs A1 and A2A ARs. In an in vitro functional assay the compound competitively antagonized the inhibition of cAMP production induced by the adenosine agonist NECA in CHO cells expressing hA3 ARs with a pA2 value of 8.1.$^{235}$

![VUF5574](image)

Fig. 24. VUF5574 as A3 AR antagonist.

Thiazole and Thiadiazole

The bicyclic system of isoquinoline and quinazoline has been replaced by several monocyclic rings.$^{165}$ Some thiazole and thiadiazole derivatives were shown to be most promising candidates for the identification of new A3 AR ligands.

The derivative N-[3-(4-methoxy-phenyl)-[1,2,4]thiadiazol-5-yl]-acetamide (A, Fig. 25) has been claimed to be the most potent A3 AR antagonist of the series, exhibiting a $K_i$ value of 0.79 nM at hA3 AR and antagonistic properties in a cAMP functional assay.$^{165}$ A series of potent and selective A3 AR antagonists have been obtained via an optimization study of compound B (Fig. 25) that revealed that a 5-(pyridine-4-yl) moiety on the 2-aminothiazole ring was optimal for enhanced receptor potency and selectivity.$^{236}$ In particular, N-[4-(3,4,5-trimethoxyphenyl)-5-pyridin-4-ylthiazol-2-yl]-acetamide C (Fig. 25) showed subnanomolar affinity at the human A3 AR as a
competitive antagonist of $[^{125}]$I–AB–MECA, binding with 1,000-fold selectivity versus the other ARs.

Fig. 25. Thiazole and Thiadiazole derivatives as A3 AR antagonists.

Binding affinity data on thiazole and thiadiazole derivatives at the hA3 AR have been subjected to QSAR analysis. This study disclosed the importance of the molecular electrostatic potential surface (Wang–Ford charges) in relation to atoms C2, C5, C7, X8 and S9 (Fig. 25), the last two playing the most important roles. Furthermore, the A3AR binding affinity increases with decreasing lipophilicity of the compounds and in the presence of short alkyl chains—methyl (Me) or ethyl (Et)—at the R position.

Pyrazoloquinolines

The binding affinities at bovine A1 and A2A ARs and at human cloned A3 ARs of some 2-arylpyrazolo[3,4-c]quinolin-4-ones along with their corresponding 4-amines and 4-substituted-amino derivatives were reported by Colotta et al. The 4-benzoylamido
derivative A (Fig. 26) displayed one of the best binding profiles of the series of A3 AR antagonists. The same group recently reported an extension of the SAR study of this class of compounds which highlighted that bulky and lipophilic acyl–amino groups at the 4 position seemed able to promote hA3 AR potency and selectivity. Selected compounds of these series were tested in an in vitro rat model of cerebral ischemia and prevented the irreversible failure of synaptic activity induced by oxygen and glucose deficiency in the hippocampus, thus confirming that potent and selective A3 AR antagonists may substantially increase the tissue resistance to ischemic damage. The synthesis and the affinity profile at ARs of a series of 2-phenyl-2,5-dihydro-pyrazolo[4,3-c]quinolin-4-ones, conceived as structural isomers of the parent 2-arylpyrazolo[3,4-c]quinoline derivatives, have also been reported. Some of the synthesized compounds showed A3 AR affinities in the nanomolar range and good selectivities, as evaluated in radioligand binding assays at hARs. In particular, substitution at the 4 position of the 2-phenyl ring with methyl, methoxy, or chlorine and the presence of a 4-oxo functionality gave good activity and selectivity (B, Fig. 26).

Fig. 26. Pyrazoloquinoline derivatives as A3 AR antagonists.

Triazoloquinoxalines

Interesting studies performed in the last decade by Colotta and coworkers highlighted that the 1,2,4-triazolo[4,3-a]quinolin-1-one moiety is an attractive scaffold for obtaining potent and selective hA3 AR antagonists. Intensive efforts in the chemical synthesis of compounds based on the systematic substitution of the 2, 4 and 6 positions of the tricyclic template, along with molecular modeling investigations...
Introduction

performed to rationalize the experimental SAR findings, led to the identification of optimal structural requirements for A3 AR affinity and selectivity. In particular, the introduction into the triazoloquinazoline moiety of a 4-oxo (A Fig. 27) or a 4-N-amido (B, Fig. 26) function affords selective and/or potent A3 AR antagonists, indicating that a C = O group (either extranuclear or nuclear) is necessary for A3 AR affinity. This suggested that the probable engagement of this site of the molecule is a hydrogen bond with the A3 AR binding site. Hindering and lipophilic acyl–amino moieties at the 4 position showed enhanced A3 AR affinity (B). Substitution of the 4 position of the 2-phenyl ring with a methoxy or a nitro group and 6-nitro substitution, as well as the combination of these substituents, afforded nanomolar A3 AR affinity and better A3 AR selectivity. 1-Oxo, 6-nitro, and 4-amino groups have been proposed to be involved in hydrogen bonds that anchor the antagonists to the binding site.

![Fig. 27. Triazoloquinazoline derivatives as A3 AR antagonists.](image)

Nucleoside-Derived A3 AR Antagonists

Based on the observation that the relative efficacy of purine nucleosides depends on structural features, new subtype-selective nucleoside antagonists of the A3 AR have been designed. One of the first such antagonists was the rigid spirolactam MRS1292 (Fig. 28, (2R,3R,4S,5S)-2-[N^6-3-iodobenzyl]adenos-9'-yl]-7-aza-1-oxa-6-oxospiro[4.4]-nonan-4,5-diol)\(^{240}\), which binds potently and selectively to the rat and human A3 ARs but does not activate these receptors, and thus acts as an antagonist. Modeling/mutagenesis of ARs has focused on distinct residues related to ligand binding and the relative efficacy of adenosine derivatives, and on a conserved Trp residue (6.48) which is involved in the activation process (termed a "rotamer switch")\(^{241}\).
Docking studies of agonists suggest that the activation pathway of the A3 AR involves a characteristic anticlockwise rotation of this residue, as viewed from the exofacial side. The docking of MRS1292 (Fig. 28) to the A3 AR model is not accompanied by rotation of this residue, as occurs with nucleoside agonists, consistent with its action as an antagonist. Moreover, the affinity and selectivity of MRS1292 occurs across species, unlike most other heterocyclic antagonists for the A3 AR reported. This allows its use in nonprimate (e.g., murine) experimental animals used as clinical models. For example, MRS1292 applied directly to the eye in mouse has been shown to be effective in reducing intraocular pressure, which may be predictive of its utility as an antiglaucoma agent. The removal of the ability of the 5'-N-alkyluronamide to donate a hydrogen bond was found to convert agonists into selective antagonists. In both the 4'-oxo and the 4'-thio series, N-methylation of an Nmethylamide (i.e., to form a dimethylamide) resulted in potent and selective A3 AR antagonists. Recently, nucleosides that are truncated at the 4' position were found to act as A3 AR antagonists. For example, (2R, 3R, 4S)-2-(2-chloro-6-(3-chlorobenzylamino)-9H-purin-9-yl) tetrahydrothiophene-3,4-diol (LJ-1416, Fig. 28) and (2R, 3R, 4S)-2-(2-chloro-6-(3-iodobenzylamino)-9H-purin-9-yl) tetrahydrothiophene-3,4-diol (LJ-1251, Fig. 28) displayed Kᵢ values of 1.66 and 4.16 nM, respectively, at the human A3 AR, with >600-fold selectivity in comparison to the A1 AR. LJ-1251 was shown to have neuroprotective properties in an ischemia model in the rat hippocampus. Truncation at the 4' position of A3 AR agonist in the (N)-methanocarba series produces potent and selective A3 AR antagonists, such as the 3-bromo derivative 1'R, 2'R, 3'S, 4'R, 5'S)-3'-[2-chloro-6-(3-bromobenzylamino)-purine]-2',3'-O-dihydroxybicyclo-[3.1.0] hexane (MRS5147) (Fig. 28) (2,900-fold selective for hA3 vs. hA1 AR) or its 3-iodo analog, MRS5127 (Fig. 28) (2,400-fold selective for hA3 vs. hA1 AR). MRS5127 displayed a KB (Schild constant) value of 8.9 nM as an antagonist of the human A3 AR in a functional assay.
Introduction

In light of the plethora of biological effects attributed to A_3 ARs, substantial efforts in medicinal chemistry have been addressed to develop antagonists for the A_3 subtype. As a result a number of molecules are in biological testing as therapeutic agents for asthma and COPD, glaucoma, cancer, and stroke^{248-250}. Use of A_3 antagonists has been patented for inhibition of tumor growth^{251}. The pre- or coadministration of pharmaceutical compositions comprising high-affinity adenosine A_3 receptor antagonists, such as MRE-3008-F20, has been patented for synergistically accentuating the response to chemotherapy consisting of taxane (e.g., paclitaxel), vinca alkaloid (e.g., vincristine), camptothecin (e.g., irinotecan), or antibiotic (e.g., doxorubicin) treatment^{252}. The claim further embodies the prevention of multidrug resistance (MDR) and targeted tumors include those expressing MDR associated protein (MRP), A_3 ARs, or P-glycoprotein, as found in leukemia, melanoma, and carcinoma of the pancreas, ovary, and lung. Moreover, MRE-3008-F20 has been also

Fig. 28. Nucleoside-Derived A_3 AR Antagonists.

Clinical Development and Patents

In light of the plethora of biological effects attributed to A_3 ARs, substantial efforts in medicinal chemistry have been addressed to develop antagonists for the A_3 subtype. As a result a number of molecules are in biological testing as therapeutic agents for asthma and COPD, glaucoma, cancer, and stroke^{248-250}. Use of A_3 antagonists has been patented for inhibition of tumor growth^{251}. The pre- or coadministration of pharmaceutical compositions comprising high-affinity adenosine A_3 receptor antagonists, such as MRE-3008-F20, has been patented for synergistically accentuating the response to chemotherapy consisting of taxane (e.g., paclitaxel), vinca alkaloid (e.g., vincristine), camptothecin (e.g., irinotecan), or antibiotic (e.g., doxorubicin) treatment^{252}. The claim further embodies the prevention of multidrug resistance (MDR) and targeted tumors include those expressing MDR associated protein (MRP), A_3 ARs, or P-glycoprotein, as found in leukemia, melanoma, and carcinoma of the pancreas, ovary, and lung. Moreover, MRE-3008-F20 has been also
Introduction

patented for the treatment of cardiac hypoxia, allergic diseases, cerebral ischemia, and cancers with high concentrations of A3 ARs\textsuperscript{253}. Other patents of A3 antagonists also concern their use for cognitive disorders, multiple sclerosis, neurodegeneration, PD, stroke, traumatic brain injury\textsuperscript{254}, asthma and COPD\textsuperscript{255-258}, glaucoma\textsuperscript{259} and arthritis\textsuperscript{260}.

A3 Adenosine Receptor: Preclinical Studies

The A3 AR plays complex roles in inflammation, with both pro- and anti-inflammatory functions being described in multiple cellular and animal models with varying roles being dictated largely by species differences\textsuperscript{64}. This receptor has also received attention in chronic inflammatory disorders of the airways. Transcript levels of A3 AR are elevated in lung biopsies of patients with asthma or COPD\textsuperscript{261}, where it is thought to localize to eosinophils. Functions attributed to A3 AR activation on human eosinophils include the inhibition of chemokine-induced migration\textsuperscript{261} and the inhibition of degranulation and superoxide anion release\textsuperscript{262}, suggesting A3 AR agonists might have utility in the treatment of asthma. In contrast to these findings in humans, pre-clinical studies in animal models suggest that A3 AR has a proinflammatory role in chronic inflammatory diseases of the airways. A3 AR is abundantly expressed on mouse eosinophils\textsuperscript{263} and \textit{in vitro} studies with mouse eosinophils confirm a role for this receptor in chemokine-induced eosinophil migration\textsuperscript{263}. However, genetic removal or pharmacological blockade of A3 AR in ADA-deficient mice results in decreased airway eosinophilia, but does not affect the degree of circulating eosinophilia, suggesting that A3 AR signaling is important in eosinophil trafficking in this model (Table 2). Moreover, a recent study demonstrated the absence of eosinophil peroxidase in the airways of A3 AR-deficient mice exposed to fibrosis inducing agent, bleomycin\textsuperscript{264}, suggesting that this receptor is needed for eosinophil degranulation \textit{in vivo}. A3 AR is also abundant on rodent mast cells and is responsible for adenosine-mediated mast cell degranulation \textit{in vitro} and \textit{in vivo} in multiple models\textsuperscript{265,266}. In addition, A3 AR is markedly elevated in mucin-producing bronchial airway epithelial cells in several models of Th2-mediated airway inflammation and airway remodeling\textsuperscript{115,121,263}, and evidence from A3 AR over expressing and knockout mice suggests that this receptor serves to enhance mucus secretion in these environments\textsuperscript{121}. These findings suggest that treatment with A3 AR
antagonists might be beneficial in models of allergic airway inflammation. The discrepancies between findings in human cellular systems and those in animal models might be due to species differences or the inability to adequately assess the function of A3 AR in vivo in humans. It is likely that the usefulness of A3 AR agonists and antagonists in the treatment of asthma and COPD will only be revealed by appropriate clinical trials with such compounds.

A3 Adenosine Receptor: Clinical Trials

As mentioned above, transcript levels of A3 AR are elevated in lung biopsies of patients with asthma or COPD where it appears to be involved in the inhibition of eosinophil chemotaxis. Because asthmatic inflammation is characterized by extensive infiltration of the airways by activated eosinophils, elevated adenosine concentrations associated with asthma might contribute to eosinophilic trafficking through modulation of A3 AR. King Pharmaceuticals published a patent application claiming a particularly potent and selective A3 AR antagonist for asthma characterized by a tricyclic xanthine derivative structure\textsuperscript{267}. As previously mentioned, a dual A2B/A3 AR antagonist, QAF 805 from Novartis, failed to attenuate bronchial hyperresponsiveness to inhaled AMP in a placebo controlled, double-blind, randomized, two-way crossover Phase Ib study of AMP sensitive asthmatics\textsuperscript{268}. A3 AR agonists such as IB-MECA and CI-IB-MECA are now advancing into Phase II clinical trials for treatments targeting diseases such as cancer, arthritis, and psoriasis. Clinical development of various A3 Adenosine Receptor (AR) agonists and antagonists is presented in Table 11. List of selected adenosine receptor antagonists in clinical development is presented in Table 12.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Drug Name</th>
<th>Molecular Target</th>
<th>Clinical Indication</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>IB-MECA (CF 101)</td>
<td>A3 AR agonists</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>2</td>
<td>CIIB-MECA</td>
<td>A3 AR agonists</td>
<td>Cancer</td>
</tr>
<tr>
<td>3</td>
<td>MRS 3558 (CF 502)</td>
<td>A3 AR agonists</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>4</td>
<td>OT 7999</td>
<td>A3 AR antagonist</td>
<td>Glaucoma</td>
</tr>
<tr>
<td>5</td>
<td>QAF 805</td>
<td>Mixed A2b/A3 AR antagonists</td>
<td>Inhibition of BHR and anti-inflammation</td>
</tr>
</tbody>
</table>
Table 12. Selected Adenosine Receptor Antagonists in Clinical Development.

<table>
<thead>
<tr>
<th>Name</th>
<th>Chemical structure</th>
<th>Phase</th>
<th>Pharmacological profile</th>
<th>Therapeutic group</th>
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<tr>
<td>Doxofylline</td>
<td><img src="image" alt="Doxofylline" /></td>
<td>Launched</td>
<td>A&lt;sub&gt;1&lt;/sub&gt;AR antagonist</td>
<td>Bronchodilator</td>
</tr>
<tr>
<td>Theophylline</td>
<td><img src="image" alt="Theophylline" /></td>
<td>Launched</td>
<td>A&lt;sub&gt;1&lt;/sub&gt;AR antagonist</td>
<td>Bronchodilator</td>
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<tr>
<td>KW-3902</td>
<td><img src="image" alt="KW-3902" /></td>
<td>Phase III</td>
<td>A&lt;sub&gt;1&lt;/sub&gt;AR antagonist</td>
<td>Agent for heart failure</td>
</tr>
<tr>
<td>N-0861</td>
<td><img src="image" alt="N-0861" /></td>
<td>Phase III</td>
<td>A&lt;sub&gt;1&lt;/sub&gt;AR antagonist</td>
<td>Agent for heart failure</td>
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<tr>
<td>FK-453</td>
<td><img src="image" alt="FK-453" /></td>
<td>Phase II</td>
<td>A&lt;sub&gt;1&lt;/sub&gt;AR antagonist</td>
<td>Agent for renal failure</td>
</tr>
<tr>
<td>FK-838</td>
<td><img src="image" alt="FK-838" /></td>
<td>Phase II</td>
<td>A&lt;sub&gt;1&lt;/sub&gt;AR antagonist</td>
<td>Agent for hypertension, diuretic</td>
</tr>
</tbody>
</table>
From the above literature review it can be observed that a wide number of compounds exerting high potency and selectivity in antagonizing the hA3 AR have been discovered. These molecules are generally characterized by a notable structural diversity, taking into account that aromatic nitrogen-containing monocyclic (thiazoles and thiadiazoles), bicyclic (isoquinoline, quinozalines, (aza)adenines), tricyclic systems (pyrazoloquinolines, triazoloquinazalines, pyrazolotriazolopyrimidines, triazolopurines, tricyclic xanthines) and nucleoside derivatives have been identified as potent and selective A3 AR antagonists. Probably due to the “enigmatic” physiological role of A3 AR, whose activation may produce opposite effects (for example, concerning tissue protection in inflammatory and cancer cells) and may produce effects that are species dependent, only a few molecules have reached preclinical investigation. Indeed, the most advanced A3 AR antagonists remain in preclinical biological testing. Among the antagonists described above, compound OT-7999 is expected to enter clinical trials for the treatment of glaucoma, while several thiazole derivatives are in development as antiallergic, antiasthmatic and/or anti-inflammatory drugs.