INTRODUCTION

Definition of Asthma

Asthma is a condition characterized by variable or intermittent obstruction of the lower airways that results in shortness of breath, wheezing, chest tightness, and cough. The American Thoracic Society and American College of Chest Physicians (1975) have offered the following definition of asthma: "A disease characterized by an increased responsiveness of the airways to various stimuli and manifested by slowing of forced expiration which changes in severity either spontaneously or as a result of therapy. Anyhow, the Expert Panel Report of the National Asthma Education Program (1991) agreed on this working definition: "Asthma is a lung disease with the following characteristics: 1) airway obstruction that is reversible (but not completely so in some patients) either spontaneously or with treatment; 2) airway inflammation; and 3) increased airway responsiveness to a variety of stimuli ". These definitions address the bronchial hyperresponsiveness and reversible obstruction of the airways in asthma.

Inflammatory Cells and Mediators

The prominence of various inflammatory cells and mediators has been demonstrated by numerous studies of asthma, yet the underlying defect remains elusive. At the cellular and molecular level, the disease is currently best described by a complex sequence of events that have artificially, but usefully, been divided into the immediate and delayed

asthmatic responses. The immediate or early response is mast cell mediated and results in rapid bronchoconstriction within 10 to 20 min of allergen exposure and resolves within 1 to 2 h. Antigen stimulation of high-affinity mast cell immunoglobulin E (IgE) receptors results in the release of preformed mediators (histamine, tryptase, heparin, chemotactic factors) and in the production of phospholipid membrane-derived mediators [prostaglandin D_2 (PGD_2), leukotriene B_4 (LTB_4), cysteinyl leukotrienes]. While histamine, some leukotrienes (LTs), and PGD_2 produce bronchoconstriction, other mediators stimulate mucous production and recruit other inflammatory cells, thereby setting the stage for the delayed response.

The delayed or late response is characterized by the involvement of eosinophils, lymphocytes, neutrophils, and alveolar macrophages. It is observed experimentally in only 50 to 60% of patients, in whom onset occurs 6 to 12 hr following antigen challenge (Finnerty and Holgate, 1989). Eosinophils have been identified as playing a central role in the pathogenesis of the late asthmatic response. They have been found in both transbronchial biopsy specimens and in bronchoalveolar larvage (BAL) fluid during the late response (De Monchy *et al.*, 1985). Like mast cells, eosinophils release preformed mediators (major basic protein, eosinophil cationic protein, peroxidase), which can produce epithelial damage, as well as membrane-derived products [LTB_4 and LTC_4, platelet activating factor (PAF), 5-hydroxy-eicosatetraenoic(HETE)], which contribute to bronchoconstriction, increased vascular permeability, mucous secretion, and further cellular chemotaxis (Finnerty and Holgate, 1989). Alveolar

macrophages and lymphocytes are also abundant in BAL specimens from asthmatic patients. Like eosinophils, they may be activated directly by antigen via low-affinity IgE receptors, and are also indirectly recruited by cellular mediators. Both macrophages and lymphocytes have been implicated as potential regulators, since both produce inflammatory cytokines (Robinson *et al.*, 1992). The late asthmatic response is complex and incompletely understood.

Histamine

Histamine or β-aminoethylimidazole is widely, if unevenly, distributed throughout the animal kingdom and is present in many venoms, bacteria and plants (Reite, 1972). Almost all mammalian tissues contain histamine in amounts ranging from less than 1 to more than 100 μg/g. Concentrations in plasma and other body fluids generally are very low, but human cerebrospinal fluid contains significant amounts (Khandelwal *et al.*, 1982). The mast cell is the predominant storage site for histamine in most tissue; the concentration of histamine is particularly high in tissues that contain large numbers of mast cells, such as skin, the mucosa of the bronchial tree, and the intestinal mucosa. Histamine is formed by decarboxylation of the amino acid L-histidine, a reaction catalyzed in mammalian tissues by the enzyme histidine decarboxylase. Pyridoxal phosphate is required as co-factor. The chief site of histamine storage in most tissues is the mast cell; in the blood, it is the basophil. These cells synthesize histamine and store it in their secretory granules with primarily proteases and heparin or chondroitin sulfate proteoglycans (Serafin and

Austen, 1987). The turnover rate of histamine in secretory granules is slow, and when tissues rich in mast cells are depleted of their stores of histamine, it may take weeks before concentrations of the autacoid return to normal. Non mast-cell sites of histamine formation or storage include cells of the epidermis, cells in the gastric mucosa, neurons within the central nervous system (CNS), and cells in regenerating or rapidly growing tissues. Histamine exerts its biological actions by combining with specific cellular receptors located on the surface membrane. The three different histamine receptors thus far characterized are designated H_1 (Ash and Schild, 1966), H_2 (Black *et al.*, 1972) and H_3 (Arrang *et al.*, 1987). All three receptor subtypes belong to the large superfamily of receptors having seven membrane spanning regions and intracellular association with G proteins (Arrang *et al.*, 1987). Histamine stimulates, or more rarely relaxes, various smooth muscles. Contraction is due to activation of H_1 receptors, coupled to phospholipase C, and their activation leads to formation of inositol-1,4,5-triphosphate (IP_3) and diacylglycerols from phospholipid in the cell membrane, IP_3 causes a rapid release of Ca^{2+} from the endoplasmic reticulum (Kamm and Stull, 1985; Somlyo *et al.*, 1988). Minute doses of histamine also will evoke intense bronchoconstriction in patients with bronchial asthma and certain other pulmonary diseases; in normal human beings the effect is much less pronounced. Although the spasmogenic influence of H_1 receptors is dominant in human bronchial muscle, H_2 receptors with dilator function also present. H_2 receptors are linked to the stimulation of adenylyl cyclase and thus to the activation of cyclic AMP-dependent protein kinase in the target cell (Kamm and Stull, 1985; Taylor

et al., 1989). Thus, histamine-induced bronchospasm *in vitro* is potentiated slightly by H_2 blockade.

Kinins

Kallidin has an additional lysine residue at the amino-terminal position and is sometimes referred to as lysyl-bradykinin. The two peptides are cleaved from α_2 globulins that are synthesized by the liver and circulate in the plasma. These precursors are termed kininogens. There are two kininogens, high molecular weight (HMW) and low molecular weight (LMW) kininogen. A number of serine proteases will generate kinins, but the highly specific proteases that release bradykinin and kallidin from the kininogens are termed kallikreins (Wachtfogel *et al.*, 1993). There are at least two distinct receptors for kinins, which have been designated B_1 and B_2 (Regoli and Barabe, 1980). The classical bradykinin receptor, now designated the B_2 receptor, selectively binds bradykinin and kallidin and is constitutively present in most normal tissue. The role of kinins in inflammation and vascular permeability is an important consideration in the pathophysiology of pulmonary disorders such as asthma. Inhalation or intravenous injection of kinins causes bronchospasm in asthmatic patients but not in normal individuals. Based on kinin-induced bronchoconstriction in guinea-pig trachea the existence of a B_3 receptor has been suggested (Farmer *et al.*, 1989; Farmer and De Siato, 1994). These recent studies indicate that the guinea-pig bronchoconstriction proposed as a B_3 receptor effect actually may represent previously unappreciated functions of the B_2 receptor (Regoli *et al.*, 1993).

Cyclo-oxygenase products

Synthesis of prostaglandins (PGs) is accomplished in a stepwise manner by a ubiquitous complex of microsomal enzymes. The first enzyme in this synthetic pathway is prostaglandin endoperoxide synthase, also called fatty acid cyclo-oxygenase. The cyclo-oxygenases have two distinct activities: an endoperoxide synthase activity that oxygenates and cyclizes the unesterified precursor fatty acid to form the cyclic endoperoxide PGG, and a peroxidase activity that converts PGG to PGH (Hamberg *et al.*, 1974). PGG and PGH are chemically unstable, but they can be transformed enzymatically into a variety of products, including prostacyclin (PGI), thromboxane (TXA), PGE, PGF, or PGD (Samuelsson *et al.*, 1975; Needleman *et al.*, 1986; Sigal, 1991). TXA_2 is formed by thromboxane synthase; TXA_2 breaks down nonenzymatically into the stable, but inactive, TXB_2. PGI_2 is formed from PGH_2 by prostacyclin synthase; it is hydrolyzed nonenzymatically to the inactive 6-keto-$PGF_{1\alpha}$. Although most tissues are able to synthesize the PGG and PGH intermediates from free arachidonate, their fate varies in each tissue and depends on the complement of enzymes that are present and on their relative abundance. For example, lung and spleen are able to synthesize the whole range of products. In contrast, platelets contain thromboxane synthase as the principal enzyme that metabolizes PGH, while endothelial cells contain primarily prostacyclin synthase. In general, PGFs and PGD_2 contract and PGEs relax bronchial and tracheal muscle. Asthmatic individuals are particularly sensitive to $PGF_{2\alpha}$, which may cause intense bronchospasm. Although both PGE_1 and PGE_2 can produce bronchodilation

such patients by aerosol, bronchoconstriction sometimes is observed (Mathe *et al.*, 1977; Spannhake *et al.*, 1981). Prostaglandin endoperoxides and TXA_2 constrict human bronchial smooth muscle. PGI_2 causes bronchodilatation in most species; human bronchial tissue is particularly sensitive, and PGI_2 antagonizes bronchoconstriction that is induced by other agents. However, as with PGEs, variable effects are produced in asthmatic patients. The diversity of the effect of prostanoids is explained by the existence of a number of distinct receptors that mediate their actions. The receptors have been named for natural prostaglandin for which they have the greatest apparent affinity and have been divided into five main types, designated DP (PGD), FP (PGF), IP (PGI_2), TP (TXA_2), and EP (PGE). The EP receptors have been further subdivided into EP_1 (smooth muscle contraction), EP_2 (smooth muscle relaxation), EP_3, and EP_4 based on physiological and molecular cloning information (Coleman *et al.*, 1994; Toh *et al.*, 1995). All prostanoid receptors identified to date are coupled to effector mechanisms through G proteins (Halushka *et al.*, 1989; Coleman *et al.*, 1994). Two second messenger systems have been associated with the action of prostanoids in platelets and smooth muscle; namely, stimulation of adenylyl cyclase (enhanced accumulation of cyclic AMP), inhibition of adenylyl cyclase (reduced accumulation of cyclic AMP), and stimulation of phospholipase C (enhanced formation of diacylglycerol (DAG) and IP_3 leading to an increase in cytosolic Ca^{2+}).

Lipoxygenase products

The metabolism of arachidonic acid by the 5-, 12- and 15-lipoxygenase results in the production of hydroperoxyeicosatetraenoic acids (HPETEs), which rapidly convert to hydroxy derivatives (HETEs) and leukotrienes. The most actively investigated leukotrienes are those produced by the 5-lipoxygenase present in inflammatory cells [polymorphonuclear neutrophils (PMNs), basophils, mast cells, eosinophils, macrophages]. This pathway is of great interest since it is associated with asthma and anaphylactic shocks. Stimulation of these cells elevates intracellular Ca^{2+}, releases arachidonate, and incorporates molecular oxygen by 5-lipoxygenase to yield the unstable epoxide LTA_4 (Borgeat and Samuelsson, 1979). This intermediate either converts to the dihydroxy LTB_4 or conjugates with glutathione to yield LTC_4 (Murphy *et al.*, 1979) which can then undergo sequential degradation of the glutathione moiety by peptidases to yield LTD_4 and LTE_4. These three products are often called cysteinyl leukotrienes or peptidoleukotrienes. LTC_4 and LTD_4 are potent bronchoconstrictors and are now recognized as the primary components of the slow-reacting substance of anaphylaxis (SRS-A), which is secreted in asthma and anaphylaxis.

Three distinct receptors for leukotrienes (LTB_4, LTC_4 and LTD_4/LTE_4) also have been identified in different tissues and cell pharmacologically and by ligand-binding techniques (Halushka *et al.*, 1989). All of these appear to activate phospholipase C and thereby

enhance formation of DAG and IP_3 leading to an increase in cytosolic Ca^{2+}.

Platelet-Activating Factor (PAF)

PAF is not stored in cells but is synthesized in response to stimulation. The precursor of PAF is 1-O-alkyl-2-acyl-glycerophosphocholine, a lipid found in high concentrations in the membranes of many types of cells. PAF is synthesized from this substrate in two steps. The first involves the action of phospholipase A_2, with the formation of 1-O-alkyl-2-lyso-glycerophosphocholine (lyso-PAF) and a free fatty acid (usually arachidonate) (Chilton *et al.*, 1984). In the second step, lyso-PAF is acetylated by acetyl coenzyme A in a reaction catalyzed by lyso-PAF acetyltransferase. The synthesis of PAF may be stimulated during antigen-antibody reactions or by a variety of antigens, including chemotactic peptides, thrombin, collagen, and other autacoids; PAF also can stimulate its own formation. Both the phospholipase and acetyltransferase are Ca^{2+}-dependent.

PAF is synthesized by platelets, neutrophils, monocytes, mast cells, eosinophils, renal mesangial cells, renal medullary cells, and vascular endothelial cells. PAF also produces effect that suggests its importance in asthma. When inhaled, it is a potent bronchoconstrictor, it promotes the accumulation of eosinophils in the lung, it causes tracheal and bronchial edema, and it stimulates the secretion of mucus. Moreover, PAF produces long-lasting bronchial hyperresponsiveness. In many cases stimulation of these receptors causes activation of phospholipase C, D, and A_2 with

resultant formation of inositol phosphates, diacylglycerol, and arachidonate (Schwertschlag and Whorton, 1988). The arachidonate released by PAF is converted to PGs, TXA_2, or LTs which may function as extracellular mediators of the effects of PAF.

Neurogenic Mechanisms

The human airways are innervated by cholinergic (parasympathetic) and adrenergic (sympathetic nerves). Stimulation of cholinergic efferents leads to bronchoconstriction and mucous secretion, due to stimulation of muscarinic M_3 receptors on the target organs. Sympathetic innervation of the lungs is sparse, being directed mainly to submucosal glands and bronchial vasculature, rather than directly to bronchial wall smooth muscle. Since the capacity of sympathetic nervous stimulation to relax airway tone depends in part on vagal tone in animal models, it may be that its effect is mediated partly by modulating parasympathetic ganglionic neurotransmission (Cabezas *et al.*, 1971). Other modulating influences on cholinergic neurotransmission of possible importance in asthma are prostaglandin and histamine release (Orehek *et al.*, 1975). Histamine is believed to owe some of its bronchoconstrictor action to stimulation of vagal tone (Gross and Skorodin, 1984). PGD_2 enhances the bronchoconstrictor effect of cholinergic agonists in asthma (Fuller *et al.*, 1986). The relative contributions of local versus vagal reflex arcs, and cholinergic versus non-cholinergic neurotransmission to airflow obstruction in asthma are not known. Afferent stimuli are transmitted in the vagus nerve, and receive input from a variety of sensory receptors. Rapidly

adapting (irritant) receptors are myelinated nerve endings lying superficially in the bronchial epithelium, which prompt coughing in response, for example, to cigarette smoke and bronchoconstriction via a vagal reflex arc.

In contrast, C-fibers are non-myelinated nerve endings. They also lie superficially in the airways and are stimulated by histamine and PGE_2 and $PGF_{2\alpha}$. Specifically, they are stimulated by inhaled bradykinin, whereas irritant receptors are not (Barnes, 1992). It is thought that C-fiber nerve terminals contain bronchoconstrictor sensory neuropeptides. The axon reflex hypothesis postulates that stimulation of C-fibers leads to nervous impulses along their axons, which can antidromically stimulate the nerve endings of enjoining C-fiber branches, leading to release of their sensory neuropeptides (Barnes, 1986). One such peptide tachykinin, neurokinin A, found in sensory airway nerves causes bronchoconstriction when inhaled by asthmatic subjects but not in normals, indicating increased sensitivity to this sensory neuropeptide in asthma (Joos *et al.*, 1987).

Recently, evidence has been gathered indicating airway innervation not corresponding to either parasympathetic or sympathetic nerves. These nerves, known as non-adrenergic, non-cholinergic (NANC) nerves, can be either bronchodilator (inhibitory) or constrictor (excitatory). NANC inhibitory nerves have been shown to innervate human bronchial smooth muscle (Richardson and Beland, 1976). Neurotransmission in NANC nerves is thought to be via neuropeptides, but this role has not been demonstrated in human airways (Finnerty and Holgate, 1989).

Reference Drugs Used in this Study

β_2-Adrenergic agonists (e.g. terbutaline)

The adrenergic agonists have several pharmacological actions that are important in the treatment of asthma, e.g. they relax airway smooth muscle and inhibit release of bronchoconstricting substances from mast cells. They may also increase mucociliary transport by increasing ciliary activity or by affecting the composition of mucous secretions. As in other tissues, the β-agonists stimulate adenylyl cyclase and exert their pharmacological actions through an elevation of intracellular adenosine cyclic AMP, activation of cyclic AMP-dependent protein kinases and phosphorylation of specific proteins in the airway tissues (Barnes, 1989). Identification of β-receptor subtypes and development of relatively specific agonists have improved the safety of adrenergic drugs. The β_2-selective adrenoceptor agonist drugs are the most widely used sympathomimetics for the treatment of asthma at the present time. They are effective after inhaled or oral administration and have a long duration of action and significant β_2-selectivity. Albuterol, terbutaline, metaproterenol, and bitolterol are available as metered-dose inhalers; bronchodilation is maximal by 30 min and persists for 3 to 4 h. Albuterol, terbutaline and metaproterenol are also prepared in tablet form. Of these agents, only terbutaline is available for subcutaneous injection. Newer β_2-selective agonists include formoterol and salmeterol. These agents were developed for an increased duration of action (12 h or more) compared with the older β_2-agonists (4 to 6 h)

(Anderson, 1993). Inhaled β_2-agonists remain the first-line drug of choice for the acute symptomatic relief of bronchospasm.

Antimuscarinic agents (e.g. atropine)

Muscarinic antagonists competitively inhibit the effect of acetylcholine at muscarinic receptors. In the airway, acetylcholine is released from efferent endings of the vagus nerve, and muscarinic antagonists can effectively block the contraction of airway smooth muscle and the increase in secretion of mucus that occurs in response to vagal activity. Antimuscarinic agents are effective bronchodilators. When given intravenously, atropine, the prototypical muscarinic antagonist, causes bronchodilation at a lower dose than that need to cause an increase in heart rate (Homer, 1998). Studies of atropine sulfate aerosol have shown that it can cause an increase in baseline airway caliber-nearly equivalent to that achieved with β-agonist agents and that this effect persists for 5 h. The bronchodilatior activity of atropine results from the blockade of muscarinic receptors on bronchial smooth muscle via specific competitive antagonism of acetylcholine. This results in both reduction of resting bronchomotor tone and inhibition of reflex-mediated bronchoconstriction in response to such triggers as mechanical stimulation, inhaled irritants, and cold dry air (Gross, 1988). Atropine and the other anticholinergic agents decrease the elevation of intracellular cyclic GMP occurring during cholinergic stimulation. The direct role of cyclic GMP in altering bronchial muscle is uncertain. An increase in cyclic GMP level in mast cells may enhance immunological release of mediators (Kaliner, 1977) and

anticholinergic agents may prevent this by blocking cholinergic receptors on the mast cell surfaces (Engelhardt, 1979).

Methylxanthines (e.g. aminophylline)

The three important methylxanthines are theophylline, theobromine, and caffeine. The theophylline preparation most commonly used for therapeutic purposes is the theophylline-ethylenediamine complex, aminophylline, which is used in intravenous formulation. (Svensson *et al.*, 1989). Several mechanisms have been proposed for the action of the methylxanthines, but none have been established as responsible for their bronchodilating effect. At high concentrations, they can be shown *in vitro* to inhibit the enzyme phosphodiesterase. Since phosphodiesterase hydrolyzes cyclic nucleotides, this inhibition results in higher concentrations of intracellular cAMP. This effect can explain smooth muscle relaxation produce by these drugs, but it is not certain that sufficiently high concentrations are achieved *in vivo* to inhibit phosphodiesterase (Homer, 1998). Another proposed mechanism is the inhibition of cell surface receptors for adenosine. These receptors modulate adenylyl cyclase activity, and adenosine has been shown to cause contraction of isolated airway smooth muscle and to enhance histamine release from cells present in the lung. The bronchodilation produced by the methylxanthines is the major therapeutic action (Mahon, 1998).

Spasmolytic agents (e.g.papaverine)

Papaverine (6,7-dimethoxy-1-veratrylisoquinoline) is an alkaloid present to the extent of about 1% in crude opium, but is, however, unrelated chemically or pharmacologically to the opioid alkaloids. It is currently considered as the prototype of nonspecific spasmolytic agents (Ferrari, 1974), and is often used as a standard drug in the screening of the activity of new antispasmodics (Nickerson, 1975). Inhibition of cyclic nucleotide phosphodiesterase, thus increasing levels of intracellular cyclic AMP, is proposed as the mechanism of action of papaverine.

Historical Background of *Clerodendrum petasites* S. Moore

Clerodendrum petasites S. Moore belongs to the family Verbenaceae. The family is large, with about 98 genera and 2,614 or more species. *C. petasites* is an erect, shrub or herb, 3 to 5 m high, often with square stem, and is widely spread over the tropics and subtropics along roadsides in hills of evergreen forest. Leaves are 15 to 20 cm long and 1.5 to 2.5 cm wide, usually opposite and rarely alternate or whorled, mostly simple. Flowers are long tubes with white color, calyx is cup shaped, typically 5-lobed (Figure 1). The fruit is a drupe (or berry) with a large kernel (Pongs-Boonrod, 1950). The Thai name of this plant is Thao yaai mom and traditionally the stem and root are used for the treatment of back, muscle and joint pain as well as to relieve asthmatic attacks and for the treatment of chronic asthma (Boonyarattanakornkit and Supawita, 1977;

Figure 1. *Clerodendrum petasites* S. Moore

Panthong *et al.*, 1986). In India a mixture of the fruits from the plant is used for regulating fertility (Lal and Lata, 1980). The root is used for various lung ailments. The Chineses use *C. petasites* for relief of fever (Pei, 1985). *C. petasites* has been found to yield several compounds including terpenoids, steroids and flavonoids (Manzoor, 1966).

Pharmacological study of the extract from the root and stem of *C. petasites* revealed that the extract did not possess antihistamine activity when tested using isolated ileum of guinea-pig and had no cardiotoxic and hypotensive action in anesthetized dogs (Mokkhasamit *et al.*, 1971).

Purpose of this Study

The purpose of this study was to evaluate the bronchodilator activity of the ethanol extract from *Clerodendrum petasites* S. Moore, in comparison with reference drugs including β_2-adrenergic agonist (terbutaline), antimuscarinic agent (atropine), methylxanthine (aminophylline) and spasmolytic agent (papaverine). The potency of test drugs was determined by comparing their median effective doses (ED_{50}) or median effective concentrations (EC_{50}). The mechanism of bronchodilator action of ethanol extract on β_2-adrenergic and muscarinic receptors was determined. In addition, the Hippocratic screening of the ethanol extract for an evaluation of behavioral changes in conscious animals was included.