

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
26 February 2004 (26.02.2004)

PCT

(10) International Publication Number
WO 2004/016270 A1

(51) International Patent Classification⁷: **A61K 31/502**,
A61P 11/06

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(21) International Application Number:
PCT/SE2003/001274

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC,
SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA,
UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(22) International Filing Date: 13 August 2003 (13.08.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0202464-4 14 August 2002 (14.08.2002) SE

(84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO,
SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM,
GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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Published:

— with international search report

*For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*



WO 2004/016270 A1

(54) Title: USE OF Itk INHIBITORS FOR THE TREATMENT OF MAST CELL-DRIVEN OR BASOPHIL-DRIVEN DISEASES

(57) Abstract: The present invention relates to the treatment of mast cell-driven or basophil-driven conditions or diseases by inhibit-
ing the activity of Inducible T cell kinase (Itk). Particular conditions or diseases that may be treated include asthma, rhinitis, COPD.

Field of the invention

Use of Itk inhibitors for the treatment of mast cell-driven or basophil-driven diseases

The present invention relates to the treatment of mast cell- or basophil-driven conditions or diseases by inhibiting the activity of Inducible T cell kinase (hereinafter referred to as Itk).

Background to the invention*Mast cells and basophils*

Mast cells and basophils each have a complex role in acquired and innate immunity.

These roles include both effector cell and, potentially, immunoregulatory activities. Mast

cells and basophils display a number of similarities but they also differ in many aspects of natural history and function, as explained below.

Both mast cells and basophils are derived from CD34⁺ hematopoietic progenitor cells, such as those present in adult bone marrow. Basophils (like other granulocytes) typically mature in the bone marrow and then circulate in the peripheral blood, from where they can then be recruited into the tissues. However mature mast cells typically do not circulate in the blood but complete their differentiation in vascularized tissues (Galli et al. 1999).

Under physiological conditions, basophils have a short life-span of a few days. IL-3 can promote the production and survival of human basophils *in vitro* and can induce basophilia *in vivo* (Galli 2000; Valent et al. 1989; Lantz et al. 1998). Unlike basophils, mast cells can be very long-lived and mast cells that are apparently mature can proliferate under certain conditions (Galli et al. 1999; Schwartz and Huff 1998).

Both mast cells and basophils express the $\alpha\beta\gamma 2$ form of the high-affinity receptor for IgE (the Fc ϵ RI receptor) on their surface.

Both cell types can be activated to secrete diverse preformed mediators, lipid mediators (synthesized *de novo*), and cytokines, after cross-linking of Fc ϵ RI-bound IgE with bivalent or multivalent antigen. Upon appropriate stimulation (for example, via the Fc ϵ RI receptor), mast cells and basophils can secrete mediators that are either preformed and granule-

associated (for example, histamine, proteoglycans and neutral proteases) or are synthesized *de novo* (for example, leukotriene C₄ [LTC₄], platelet activating factor [PAF] and [in mast cells only] prostaglandin D₂ [PGD₂]). Furthermore, mouse or human mast cells represent potential sources of many cytokines with effects in inflammation, immunity, haematopoiesis, tissue remodelling and diverse other biological processes (for example, IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-16, TNF- α , bFGF, VPF/VEGF, TGF- β and several C-C chemokines, including MIP-1 α and MCP-1). By contrast, the spectrum of basophil-derived cytokines appears to be more limited but includes IL-4 and IL-13 (Brunner et al. 1993; MacGlashan et al. 1994).

Mast cells and basophils are not only important effector cells in acute IgE-associated allergic reactions but may also contribute significantly to the expression of aspects of acquired immune responses that develop over hours (for example, late-phase reactions [LPRs]) or days to weeks (for example, chronic allergic inflammation) (Galli et al. 1999; Schwartz and Huff 1998; Metcalfe et al. 1997; Galli 2000). Mast cells and basophils may also mediate immunoregulatory functions, both through their ability to produce certain cytokines and by other mechanisms (Galli et al. 1999; Schwartz and Huff 1998; Metcalfe et al. 1997; Galli 2000; Mecheri and David 1997).

Studies in reconstituted mast cells, mast-cell-deficient mice and correlative analyses in humans indicate that mast cells can contribute to leukocyte infiltration, tissue remodelling and long-term functional changes in the context of IgE-associated acquired immunity. Basophils can be prominent in the leukocytic infiltrates elicited at sites of IgE-associated late-phase responses in the skin, nose and airways of humans, and may contribute to the expression of acquired immunity to certain nematodes or to the feeding of ectoparasites such as ticks.

Beyond influencing immune responses via the secretion of cytokines, *in vitro* evidence has suggested that mast cells and/or basophils can function as antigen-presenting cells (Mecheri and David 1997) and represent sources of co-stimulatory activity.

Animal studies have demonstrated that mast cells are essential for virtually all of the augmented vascular permeability and tissue swelling that are associated with IgE-dependent passive cutaneous anaphylaxis reactions (Galli et al. 1999). Several findings indicate that mast cells are critical for the immediate phase of IgE-associated 'Type I'

reactions also in humans (Galli et al. 1999; Schwartz and Huff 1998; Metcalfe et al. 1997; Galli 2000; Schwartz 1994). However, in many allergic individuals, the immediate reaction to cutaneous antigenic challenge is followed 4-8 hours later by persistent swelling and leukocyte infiltration (i.e. the late phase response) (Galli et al. 1999; Schwartz and Huff
5 1998; Metcalfe et al. 1997; Galli 2000). Many of the clinically significant consequences of IgE-dependent reactions, both in the respiratory tract and in the skin, are now thought to reflect the actions of the leukocytes recruited to these sites during LPRs rather than the direct effects of the mediators released by mast cells at early time points after antigen challenge (Galli et al. 1999; Schwartz and Huff 1998; Metcalfe et al. 1997; Galli 2000).

10 Several lines of evidence, derived from both clinical and animal studies, indicate that mast cell activation can contribute to the leukocyte infiltration associated with late phase responses. The leukocytes recruited to sites of LPRs can include basophils, eosinophils, neutrophils, lymphocytes and monocytes/macrophages. All of these cells may influence
15 the reactions by providing additional proinflammatory mediators and cytokines, with the relative importance of the various potential effector cells varying depending on the individual circumstances (Galli et al. 1999; Schwartz and Huff 1998; Metcalfe et al. 1997; Galli 2000).

20 The release of cytokines and other mediators from mast cells and basophils has the potential to influence many aspects of the pathophysiology at sites of allergic diseases, including some of the chronic changes (for example, airway hyper-reactivity, connective tissue changes and enhanced mucus production) that are associated with these disorders (Galli et al. 1999; Schwartz and Huff 1998; Metcalfe et al. 1997; Galli 2000; Gordon and
25 Galli 1994). Studies with adoptively reconstituted mast-cell-deficient mice having genetically manipulated mast cell lines have shown that mast cells can contribute significantly to the development of at least three of the long-term features of asthma in mouse models of the disorder: allergen-induced bronchial hyper-reactivity to cholinergic stimulation (Kobayashi et al. 2000; Williams and Galli 2000), eosinophil infiltration, and
30 increased numbers of proliferating cells in the airway epithelium (Williams and Galli 2000). Furthermore, IL-4 which can be released from either T cells or basophils recruited to sites of allergic inflammation, as well as from at least some mast cells, may contribute to the local enhancement of mast cell effector function and proliferation (Bischoff et al. 1999; Lorentz et al. 2000). It has been demonstrated that not only mast cells but also basophils
35 accumulate in nasal epithelium after allergen challenge (Klein 2000).

In addition to the Th2 cells, mast cells also play an important role in initiating and maintaining the allergic response in asthma and allergic rhinitis (Holgate 2000). Exposure to allergens trigger a range of cellular events in the mast cell including release of pre-formed granule-held mediators such as histamine and tryptase, synthesis and release of newly formed mediators such as PGD₂ and LTC₄, and the stimulation of a wide range of pro-inflammatory cytokines and chemokines such as TNF α , IL-4, IL-5, IL-6, IL-8, IL-13 and GM-CSF (Bingham and Austen, 2000). Thus the mast cell cannot only initiate an immediate reaction but can also initiate and co-ordinate the later inflammatory response. This process has also been shown to occur in seasonal allergic rhinitis (Bentley *et al* 2000).

In summary, a variety of mediators and cells contribute to the allergic response. IgE and mast cells have been implicated both in the acute and late phases of an allergic response. The T cell, and in particular the Th2 cell, is thought to orchestrate the allergic responses through the production of cytokines such as IL-4, IL-5, IL-9 and IL-13. Together these Th2-derived cytokines will drive the production of allergen-specific IgE (IL-4), the IL-5 together with IL-3 regulates the eosinophil component, and the IL-3 and IL-4 will also be involved in the differentiation of basophils and mast cells. Recent data implicate IL-13, and potentially IL-9, to be key players in regulating the hyper-reactivity and mucus production seen in asthmatic patients.

At the effector end of the allergic reaction is the acute-phase classical hypersensitivity reaction mediated by cross-linking of allergen specific IgE bound to the Fc ϵ R1 on mast cells and basophils. This induces an immediate release of granule-associated mediators such as histamine and leukotrienes, followed by subsequent release of chemokines and cytokines involved in the recruitment of cells responsible for the late phase reactions. The late phase reaction is characterized by the influx of eosinophils and activated Th2 cells, and is manifested by oedema and mucus production. The late phase also triggers the influx of other inflammatory cells such as monocytes and neutrophils, which contribute to a sustained general inflammation.

Preventing activation of mast cells and/or basophils has for a long time been considered an attractive strategy for treatment of allergic symptoms. Such activation may occur either by IgE-dependent or IgE-independent stimuli that leads to the secretion of several

mediators including histamine, heparin, pro-inflammatory cytokines and proteolytic enzymes. A variety of different mechanisms and targets have been suggested as suitable to intervene in the FcεRI mediated mast cell / basophil activation and degranulation process (Oliver *et al* 2000). These include, among others, inhibition of the two kinases
5 Syk and Lyn located proximally to the FcεRI receptor. Lyn mediates the phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) of the β and γ subunits of the tetrameric (αβγ₂) FcεRI. The dually phosphorylated γ subunit ITAM is needed for the recruitment and activation of Syk. Syk, when activated, is responsible for the downstream propagation of the signal (Jouvin 1994). Data from Syk knock-out mice shows that mast
10 cells from these mice fail to degranulate (Costello *et al*, 1996). However, Syk is also expressed in platelets and studies of Syk-/- mice showed that this enzyme is critical for effective blood coagulation and vascular integrity (Law *et al*, 1999) and hence may not provide an attractive drug target. Studies of Lyn-/- mice showed accumulation of plasma cells in spleen as they age, resulting in elevated levels of serum IgM and
15 glomerulonephritis due to the presence of immune complexes containing auto-reactive antibodies (Wang *et al*, 1996). Therefore, inhibiting Lyn for therapeutic purposes may present a significant challenge.

Mast cell stabilizers are currently being used to reduce the release of histamine and other
20 inflammatory mediators by stabilizing mast cells, the mechanism of action has yet to be determined (Spector 1999).

The use of recombinant anti-IgE antibodies blocking the binding of circulating IgE to the FcεRI has been reported to prevent mast cell / basophil degranulation (MacGlashan *et al*,
25 1997). Reduced serum IgE levels and basophil FcεRI expression levels accompany the symptomatic improvements in allergic rhinitis and asthmatic symptoms observed.

Itk

Itk (Inducible T cell kinase) is a 72 kDa kinase expressed in the cytoplasm of T cells, NK
30 cells, mast cells and basophils, but not in other immune cells or outside the haematopoietic system. Itk is also known as Interleukin-2-inducible T cell kinase, Emt (expressed mainly in T cells) or Tsk (T cell specific tyrosine kinase).

Itk is a member of the Tec-family of cytosolic protein tyrosine kinases. In mammals, this family also includes Btk (Bruton's tyrosine kinase), Tec, Bmx, and Txk. The Tec-family kinases regulate various immune cell functions that integrate signals given by the other cytosolic tyrosine kinases as well as by serine/threonine kinases, lipid kinases, and small G proteins (Yang et al 2000).

Tec-family kinases have the following general structure: a N-terminal pleckstrin-homology (PH) domain, a Tec-homology domain that includes a Btk motif and one or two proline-rich (PR) motifs, a SH3 domain, a SH2 domain and a c-terminal catalytic (SH1) domain. These kinases are expressed almost exclusively in hematopoietic tissues. Tec and Bmx have also been detected in endothelial cells. The cellular distribution differs for each Tec-family member. For example, Itk is expressed by T cells, NK cells, mast cells and basophils, whereas Btk is expressed by all hematopoietic cells except T cells. Thus hematopoietic cells may express one or several Tec-family kinases. For example T cells express Itk, Tec and Txk, and mast cells express Btk, Itk and Tec.

The gene for Itk (the *itk* gene) is located on human chromosome 5q in a cluster of genes associated with a Th2 type immune response (for example, IL-4 and IL-5). No disease association has been described for the *itk* gene. In contrast, the gene for Btk has been very thoroughly studied due to its association with X-linked agammaglobulinemia (XLA). XLA patients are virtually devoid of mature B cells and their Ig levels are strongly reduced. The B cell deficiency is due to a defective expansion of the pre-B cell pool. Btk^{-/-} mice, and *xid* mice carrying a mutation which results in the substitution of Arg with Cys at residue 28 in the Btk protein, have about half the number of B cells as compared to normal mice (Smith et al 2001). Mast cell development is normal in Btk^{-/-} mice, but bone-marrow derived mast cells (BMMCs) exhibit a reduction in antigen-induced histamine release.

In T and B cells, signalling through T cell receptors and B cell receptors leads to activation of Itk and Btk, respectively. Downstream of Itk and Btk a number of different messengers are engaged, including scaffolding proteins (SLP-76, LAT, SLP-65), Src kinases, MAP kinases, and PI3-K. These events are followed by PLC- γ activation that leads to IP3 generation and sustained Ca²⁺ flux, and subsequently activation of transcription factors. PLC- γ 1 has been suggested as a direct substrate for Itk (Miller and Berg 2002). In T cells, Itk (and Tec) may also mediate signalling through the CD28 co-receptor.

Published studies, mainly involving Btk^{-/-} animals and their mast cells, have strongly suggested that Btk is the Tec-family kinase mediating signalling from the FcεRI. No other Tec family kinase has yet been shown to have a critical role in the signalling pathways downstream of FcεRI. .

Signalling from Tec-family kinases can also be regulated by PH domain-mediated plasma membrane localization, and by Src-family-mediated phosphorylation of critical tyrosine residues.

In T cells, it is known that Itk is involved in the signalling cascade downstream of the T cell receptor (TcR), and is also associated with the co-stimulatory molecule CD28. Most of current knowledge on the role of Itk in T cell signalling has been generated in Itk-deficient knockout mice (Itk^{-/-}). Such animals have a decreased number of mature thymocytes and a reduced response to anti-CD3 stimulation, but no general T cell defect, as demonstrated by their intact response to PMA/ionomycin stimulation (Liao and Littman 1995). From studies using Itk^{-/-} mice, it has been proposed that Itk is required for Th2 but not Th1 cell development. This was demonstrated in the *N. brasiliensis* and *L. major* infection models where the Itk^{-/-} animals are protected in the Leishmania model indicating an intact Th1 response, whereas they are susceptible to infection with *N. Brasiliensis* that requires an intact Th2 response for resolution of the infection (Fowell et al 1999). Thus, in T cells Itk apparently modulates the TcR signal in a way that will allow Th1 but not Th2 differentiation. However, the functional role of Itk in mast cell development and function has not been previously identified or described.

Itk inhibitors and their uses

The international patent application published as WO 02/50071 discloses particular thiazolyl compounds that are inhibitors of the Tec-family kinases and may be used as immunosuppressive, anti-inflammatory, anti-allergic and anti-cancer agents. The compounds are described as inhibitors of Itk and other Tec-family kinases including Btk, Txk, Tec and Bmx.

The international patent application published as WO 01/25220 discloses particular triazine compounds that are inhibitors of a wide range of phosphoryl transferases or

kinases, including the Tec-family kinases (Bmx, Btk, Itk, Tec and Txk). The compounds are described as useful for treating a wide range of diseases.

The international patent application published as WO 01/66107 describes treatment of conditions associated with cytokine production by administering Tec-family kinase inhibitors, preferably inhibitors of Btk, Tec, Bmx or Txk and most preferably of Btk.

The international patent application published as WO 02/34899 describes methods of screening for compounds that modulate the activity of truncated Tec-family kinase polypeptides, and therapeutic uses of the compounds identified.

Description of the invention

We have identified the critical role of Itk in regulating important mast cell and basophil functions. The activity of mast cells or basophils may be blocked through inhibition of Itk. Thus Itk inhibitors may be used as pharmaceutical agents for the treatment or prevention of mast cell-driven or basophil-driven conditions or diseases.

In particular we have identified Itk as a target for inhibiting several key events in both acute and late phase allergic reactions common to allergic rhinitis and asthma.

We have demonstrated that inhibiting Itk activity in primary human mast cells and/or basophils leads to reduced levels of histamine release after stimulation through FcεRI. Itk inhibitors are able to inhibit histamine release in αIgE stimulated primary human basophils and in human lung mast cells. This effect is not observed when using Btk selective inhibitors. Thus Itk is the critical, rate-limiting enzyme that controls signalling from the FcεRI and drives the downstream responses.

Our findings indicate that an Itk inhibitor should reduce both the acute and late phase responses of an allergic reaction, as seen in both rhinitis and asthma and other conditions or diseases. Thus an Itk inhibitor may be used as a pharmaceutical agent to treat either the acute phase or the late phase responses of an allergic reaction. It is also possible to treat both the acute phase and the late phase responses with a single pharmaceutical agent (the Itk inhibitor), avoiding the need for polytherapy. Thus a single drug (Itk

inhibitor) may replace the use of two separate drugs (such as an anti-histamine drug plus a glucocorticosteroid drug).

5 In a first aspect of the invention, we provide a method of treating or preventing a mast cell-driven or a basophil-driven condition or disease which comprises administering a therapeutically effective amount of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof to a human or mammal suffering from or susceptible to the condition or disease.

10 Preferably we provide a method of treating or preventing a mast cell-driven condition or disease.

In a further aspect of the invention, we provide the use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof for the treatment or prevention of a mast
15 cell-driven or a basophil-driven condition or disease. The Itk inhibitor (or its salt or ester) is used to inhibit mast cell responses and/or basophil responses, including activation and degranulation.

20 Preferably we provide the use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof for the treatment or prevention of a mast cell-driven condition or disease.

In particular we provide the use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof in the manufacture of a medicament for the treatment or prevention of a
25 mast cell-driven or a basophil-driven condition or disease.

Preferably we provide the use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof in the manufacture of a medicament for the treatment or prevention of a
mast cell-driven condition or disease.

30 The treatment or prevention of a condition or disease covers all forms of medical therapy, including prophylactic, diagnostic and therapeutic regimens carried out *in vivo* or *ex vivo* on humans or other mammals. Prevention of a condition or disease includes reducing the risk of that condition or disease. Humans or mammals susceptible to a condition or disease are those at risk of suffering from the condition or disease.

Prophylaxis is expected to be particularly relevant to the treatment of persons who have suffered a previous episode of, or are otherwise considered to be at increased risk of, the condition or disease in question. Persons at risk of developing a particular condition or disease generally include those having a family history of the disease or condition, or
5 those who have been identified by genetic testing or screening to be particularly susceptible to developing the condition or disease.

Mast cell-driven and basophil-driven conditions or diseases include allergic, inflammatory, autoimmune, proliferative and hyper-proliferative diseases. Particular types and specific
10 examples of conditions and disease are discussed below.

Allergic, inflammatory or auto-immune conditions or diseases of the respiratory tract include: reversible obstructive airway diseases such as asthma (for example, bronchial, allergic, intrinsic asthma, extrinsic and chronic asthma), and associated manifestations of
15 the disease (late responses, hyper-responsiveness); farmer's lung and related diseases; fibrosis; idiopathic interstitial pneumonia; chronic obstructive airway disease (also known as chronic obstructive pulmonary disease or COPD); bronchoectasis; cystic fibrosis; eosinophilic pneumonias; adult respiratory distress syndrome (ARDS); emphysema; and alveolitis (for example cryptogenic fibrosing alveolitis).

20 Allergic, inflammatory or auto-immune conditions or diseases in the nose include all conditions or diseases characterised by inflammation of the nasal mucosal membrane such as acute rhinitis, allergic rhinitis, chronic rhinitis including caseosa, hypertrophic rhinitis, rhinitis purulenta, and rhinitis sicca, rhinitis medicamentosa, membranous rhinitis
25 including croupous, fibrinous and pseudomembranous rhinitis, scrofulous rhinitis, seasonal rhinitis including rhinitis nervosa (hay fever) and vasomotor rhinitis. Of particular interest are allergic rhinitis and seasonal rhinitis including rhinitis nervosa (hay fever). Suitable conditions or diseases in the nose also include nasal polyps and allergic manifestations of nasopharynx.

30 Allergic, inflammatory or auto-immune conditions or diseases of the eye include conjunctivitis (allergic, acute, vernal, of hay fever, chronic) and inflammation disorders of the eyelids, cornea, uveal tract and retina.

Allergic, inflammatory and auto-immune conditions or diseases of the gastrointestinal tract include food allergy and food intolerance, ulcerative colitis, Crohn's disease, irritable bowel disease, gastric ulcers, and food related allergic diseases which have symptomatic manifestations remote from the gastrointestinal tract (for example migraine, rhinitis and
5 eczema).

Allergic, inflammatory or auto-immune conditions or diseases of the skin include psoriasis, atopic dermatitis, contact dermatitis/dermatitis herpetiformis, erythema nodosum, urticaria, cutaneous eosinophilias, acne, Alopecia areata, eosinophilic fasciitis
10 dermatomyositis, photoallergic sensitivity and periodontal disease.

Allergic, inflammatory or auto-immune conditions or diseases of the joints and connective tissue include osteoarthritis, systemic lupus erythematosus, vasculitis, Wegener's granulomatosis, polyarthritis nodosa, bursitis, tendonitis, gout, Behcet's syndrome,
15 ankylosing sponditis, Reiter's syndrome and psoriatic arthritis.

Allergic, inflammatory, and auto-immune conditions or diseases of the circulatory system include arteroma, reperfusion injury (such as angioplasty), myocardial infarction, thrombosis, and vascular and tissue damage caused by ischaemia or injury.
20

Allergic, inflammatory, and auto-immune conditions or diseases of the central nervous system (CNS) include Parkinson's disease, Alzheimers and other dementias, stroke and subarachnoid heamorrhage.

25 Inflammatory conditions or diseases of the liver include hepatitis, cirrhosis and glomerulonephritis.

Allergic, inflammatory, and auto-immune conditions or diseases of the bladder and urogenital tract include cystitis.
30

In a preferred aspect of the invention, we provide a method for treating or preventing a reversible obstructive airway disease (especially asthma) which comprises administering a therapeutically effective amount of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof to a human or mammal suffering from or susceptible to the disease. We
35 further provide the use of an Itk inhibitor or a pharmaceutically acceptable salt or ester

thereof for the treatment or prevention of a reversible obstructive airway disease (especially asthma). We also provide the use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof in the manufacture of a medicament for the treatment or prevention of a reversible obstructive airway disease (especially asthma).

5

In another preferred aspect of the invention, we provide a method for treating or preventing rhinitis which comprises administering a therapeutically effective amount of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof to a human or mammal suffering from or susceptible to rhinitis. We further provide the use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof for the treatment or prevention of rhinitis. We also provide the use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof in the manufacture of a medicament for the treatment or prevention of rhinitis. (In this context, the term rhinitis includes all the various types of rhinitis discussed above. Of particular interest are allergic rhinitis and seasonal rhinitis including rhinitis nervosa or hay fever).

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In a further preferred aspect of the invention, we provide a method for treating or preventing chronic obstructive pulmonary disease which comprises administering a therapeutically effective amount of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof to a human or mammal suffering from or susceptible to chronic obstructive airway disease. We further provide the use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof for the treatment or prevention of chronic obstructive pulmonary disease. We also provide the use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof in the manufacture of a medicament for the treatment or prevention of chronic obstructive pulmonary disease.

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An Itk inhibitor for use in the invention is any compound having Itk inhibitory activity. The Itk inhibitor is a direct inhibitor of the Itk enzyme. The level of Itk inhibitory activity must be sufficient for the Itk inhibitor to possess useful therapeutic properties. When tested in the *in vitro* assay described in the Examples, the Itk inhibitor preferably gives an IC₅₀ value for inhibition of Itk activity of less than 5 μ M (most preferably less than 1 μ M).

30

The Itk inhibitor may be a compound of any chemical structure that possesses the necessary Itk inhibitory activity. For example, suitable Itk inhibitors include the compounds described in the Examples.

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The Itk inhibitor may be a racemate or may exist in enantiomeric or diastereoisomeric forms or mixtures thereof.

5 Preferably the Itk inhibitor is a selective Itk inhibitor (that is, an inhibitor that is more active against Itk than it is against Btk). A selective Itk inhibitor for use in the invention is any compound which has Itk inhibitory activity that is significantly greater than its Btk inhibitory activity, preferably selectivity is over 50-fold against Btk (ie the Itk inhibitor gives an IC_{50} value for inhibition of Btk activity *in vitro* which is more than 50-fold greater than its IC_{50} value for inhibition of Itk activity *in vitro*). Most preferably selectivity of the Itk inhibitor is 10 also over 10-fold (preferably over 20-fold) against Syk and/or over 10-fold (preferably over 50-fold) against Lyn. (The kinases Syk and Lyn are believed to have a regulatory role in the signalling pathway directly downstream of the Fc ϵ RI receptor).

15 Preferably the selective Itk inhibitor gives an IC_{50} value for inhibition of Itk activity of less than 5 μ M (most preferably less than 1 μ M) when tested in the assay described in the Examples.

For the above mentioned therapeutic indications, the dose of the Itk inhibitor to be 20 administered will depend on the compound employed, the disease being treated, the mode of administration, the age, weight and sex of the patient. Such factors may be determined by the attending physician. However, in general, satisfactory results are obtained when the compounds are administered to a human at a daily dosage of between 0.1 mg/kg to 100 mg/kg (measured as the active ingredient).

25

The Itk inhibitor may be used on its own, or in the form of appropriate pharmaceutical formulations comprising the Itk inhibitor in combination with a pharmaceutically acceptable diluent, adjuvant or carrier. Particularly preferred are compositions not containing material capable of causing an adverse reaction, for example, an allergic reaction. Conventional 30 procedures for the selection and preparation of suitable pharmaceutical formulations are described in, for example, "Pharmaceuticals - The Science of Dosage Form Designs", M. E. Aulton, Churchill Livingstone, 1988.

The Itk inhibitors may be administered topically, for example, to the lungs and/or the airways, in the form of solutions, suspensions, HFA aerosols or dry powder formulations, for example, formulations in the inhaler device known as the Turbuhaler®; or systemically, for example, by oral administration in the form of tablets, pills, capsules, syrups, powders or granules; or by parenteral administration, for example, in the form of sterile parenteral solutions or suspensions; or by rectal administration, for example, in the form of suppositories.

10 Dry powder formulations and pressurized HFA aerosols of the Itk inhibitor may be administered by oral or nasal inhalation. For inhalation the compound is desirably finely divided. The finely divided compound preferably has a mass median diameter of less than 10 μm , and may be suspended in a propellant mixture with the assistance of a dispersant, such as a C_8 - C_{20} fatty acid or salt thereof, (for example, oleic acid), a bile salt, a
15 phospholipid, an alkyl saccharide, a perfluorinated or polyethoxylated surfactant, or other pharmaceutically acceptable dispersant.

The Itk inhibitor may also be administered by means of a dry powder inhaler. The inhaler may be a single or a multi dose inhaler, and may be a breath actuated dry powder inhaler.

20 One possibility is to mix the finely divided Itk inhibitor with a carrier substance, for example, a mono-, di- or polysaccharide, a sugar alcohol, or an other polyol. Suitable carriers are sugars, for example, lactose, glucose, raffinose, melezitose, lactitol, maltitol, trehalose, sucrose, mannitol; and starch. Alternatively the finely divided Itk inhibitor may be coated by another substance. The powder mixture may also be dispensed into hard
25 gelatine capsules, each containing the desired dose of the active compound.

Another possibility is to process the finely divided powder into spheres which break up during the inhalation procedure. This spheronized powder may be filled into the drug
30 reservoir of a multidose inhaler, for example, that known as the Turbuhaler® in which a dosing unit meters the desired dose which is then inhaled by the patient. With this system the active Itk inhibitor, with or without a carrier substance, is delivered to the patient.

For oral administration the Itk inhibitor may be admixed with an adjuvant or a carrier, for
35 example, lactose, saccharose, sorbitol, mannitol; a starch, for example, potato starch,

corn starch or amylopectin; a cellulose derivative; a binder, for example, gelatine or polyvinylpyrrolidone, and/or a lubricant, for example, magnesium stearate, calcium stearate, polyethylene glycol, a wax, paraffin, and the like, and then compressed into tablets. If coated tablets are required, the cores, prepared as described above, may be
5 coated with a concentrated sugar solution which may contain for example, gum arabic, gelatine, talcum, titanium dioxide, and the like. Alternatively, the tablet may be coated with a suitable polymer dissolved in a readily volatile organic solvent.

For the preparation of soft gelatine capsules, the Itk inhibitor may be admixed with for
10 example, a vegetable oil or polyethylene glycol. Hard gelatine capsules may contain granules of the compound using either the above mentioned excipients for tablets. Also liquid or semisolid formulations of the drug may be filled into hard gelatine capsules.

Liquid preparations for oral application may be in the form of syrups or suspensions, for
15 example solutions containing the Itk inhibitor, the balance being sugar and a mixture of ethanol, water, glycerol and propylene glycol. Optionally such liquid preparations may contain colouring agents, flavouring agents, saccharine and/or carboxymethylcellulose as a thickening agent or other excipients known to those skilled in art.

20 The Itk inhibitor may also be administered in conjunction with other compounds used for the treatment of the above conditions or diseases.

In a further aspect of the invention, we provide methods for identifying and/or developing Itk inhibitors as useful pharmaceutical agents.

25

To identify Itk inhibitors that may be useful pharmaceutical agents, it is advantageous to screen for and select compounds that inhibit Itk activity in mast cells or basophils using appropriate *in vitro* or *in vivo* assays. In particular, it is advantageous to screen for and select compounds that have the potential to inhibit Itk activity in these cells *in vivo* (for
30 example, because they are targeted to these cells).

It may also be advantageous to develop Itk inhibitors by improving their ability to inhibit Itk activity in mast cells or basophils (for example, by developing compound features or other mechanisms that help to target the inhibitors to these cells).

35

Appropriate assays that may be useful in identifying and/or developing Itk inhibitors (particularly selective Itk inhibitors) include one or more assays selected from those described in the Examples: Itk LANCE TRF assay, Btk LANCE TRF assay, Lyn LANCE TRF assay, Syk assay, Basophil Histamine Release assay (algE induced), Basophil
5 Leukotriene C4 (LTC4) Release assay (algE induced), Basophil Interleukin-4 (IL-4) Release assay (algE induced), Lung Mast Cell Histamine Release assay (algE induced). One or more of the following assays may be particularly useful in identifying and/or developing Itk inhibitors that act in basophils or mast cells: Basophil Histamine Release assay (algE induced), Basophil Leukotriene C4 (LTC4) Release assay (algE induced),
10 Basophil Interleukin-4 (IL-4) Release assay (algE induced), Lung Mast Cell Histamine Release assay (algE induced).

The present invention will now be described with reference to the following non-limiting Examples.

15

EXPERIMENTAL DETAILS FOR THE EXAMPLES

Preparation of compounds

20 *General methods*

All reactions were performed in dried glassware in an argon atmosphere at room temperature, unless otherwise noted. All reagents and solvents were used as received. Merck Silica gel 60 (0.040-0.063 mm) was used for preparative silica gel chromatography. A Kromasil KR-100-5-C18 column (250 x 20 mm, Akzo Nobel) and mixtures of
25 acetonitrile/water at a flow rate of 10 ml/min was used for preparative HPLC. Reactions were monitored at 254 nm by analytical HPLC, using a Kromasil C-18 column (150 x 4.6 mm) and a gradient (containing 0.1% trifluoroacetic acid) of 5 to 100% of acetonitrile in water at a flow rate of 1 ml/min. Evaporations of solvents were performed under reduced pressure using a rotary evaporator at a maximum temperature of 60°C. Products were
30 dried under reduced pressure at 40 °C.

¹H NMR spectra were recorded on a Varian Inova-400 or Unity-500+ instrument. The central solvent peak of chloroform-*d* (δ_{H} 7.27 ppm), dimethylsulfoxide-*d*₆ (δ_{H} 2.50 ppm) or methanol-*d*₄ (δ_{H} 3.35 ppm) were used as internal references. Low resolution mass spectra obtained on a Hewlett Packard 1100 LC-MS system equipped with a APCI ionisation
35 chamber.

*Preparation of Compound A5***5-Bromo-3-(4-morpholin-4-ylphenyl)-2-phenyl-1H-pyrrolo[2,3-b]pyridine**

2-(4-morpholin-4-ylphenyl)-1-phenylethanone (150 mg, 0.53 mmol) and (5-bromo-pyridin-2-yl)-hydrazine (100 mg, 0.53 mmol) in benzene (40 mL) containing acetic acid (0.4 mL) was heated at reflux temperature for 20 h. Water was continuously distilled off using a Dean-Starch trap. Crude, impure title compound was crystallized from the reaction mixture at 8 °C. This material was heated in an inert atmosphere at 230°C for 7 min and then partitioned between toluene and water. The toluene phase was washed with water and brine and then evaporated. The residue was chromatographed (SiO₂; EtOAc-heptane 1:3) to give the title compound (67 mg, 29 %).

¹H NMR (400 MHz, DMSO-*d*₆): δ 12.21 (1H, s); 8.24 (1H, d); 7.88 (1H, d); 7.27-7.44 (7H, m); 6.90 (2H, d); 3.70 (4H, dd); 3.14 (4H, dd).
APCI-MS *m/z*: 434.1/436.1 [MH⁺]

*Preparation of Compound A14***5-Bromo-3-[4-(2-morpholin-4-ylethoxy)phenyl]-2-phenyl-1H-pyrrolo[2,3-b]pyridine trifluoroacetate**

A mixture of 4-(5-bromo-2-phenyl-1H-pyrrolo[2,3-b]pyridin-3-yl)phenol (100 mg, 0.27 mmol), NaH (60% susp. in mineral oil, 45 mg, 1.25 mmol) and DMF (2 ml) was heated at 60°C for 30 min. Mixture of 4-(2-chloroethyl)morpholine hydrochloride (53 mg, 0.28 mmol), NaH (60% susp. in mineral oil, 15 mg, 0.42 mmol) and DMF (500 µl) was added and the reaction mixture was further stirred at 60°C for 75 min. Water (1 ml) and AcOH (200 µl, 3.5 mmol) were added and product was purified with preparative HPLC (RP-18, acetonitrile/water/trifluoroacetic acid gradient from 10:90:0.1 to 95:5:0.1) to give the title compound (59 mg, 36%).

¹H NMR (400 MHz, CD₃CN): δ 8.31 (1H, d); 8.00 (1H, d); 7.28-7.54 (7H, m); 7.01-7.12 (2H, m); 4.36-4.50 (2H, m); 3.84-4.26 (2H, m); 3.52-3.68 (4H, m); 3.20-3.45 (4H, m).
APCI-MS *m/z*: 478.0/480.0 [MH⁺].

General method for preparation of 2-arylbenzothiazole-6-carboxamides

A mixture of 4-amino-3-mercaptobenzoic acid (5 mmol) and an aromatic aldehyde 5.1 mmol) were dissolved in acetic acid (5 ml) and nitrobenzene (15 ml). The solution was heated at 185°C for 15 min. After cooling the reaction mixture was made alkaline by the addition of 1 M NaOH and extracted with EtOAc. Following acidification with HCl (conc.), the water phase was extracted with EtOAc. Drying and evaporation of the solvent gave the 2-arylbenzothiazole-6-carboxylic acid.

A mixture of 2-arylbenzothiazole-6-carboxylic acid (1 mmol), HATU (1.2 mmol) and *N,N*-diisopropylethylamine (5 mmol) were dissolved in 20 ml of DMF. After stirring for 5 min at rt, the amine (2 mmol) was added and the mixture stirred at rt overnight. CH₂Cl₂ (50 ml) was added and the solution was washed with 1 M NaHCO₃ and water. Drying and evaporation of the solvent yielded the crude title product which was purified by column chromatography (SiO₂; MeOH-CH₂Cl₂, 5:95).

15

*Preparation of Compound B3***2-(4-chloro-2-hydroxyphenyl)-N-(2-pyrrolidin-1-ylethyl)-1,3-benzothiazole-6-carboxamide**

Compound B3 was synthesized following the protocol for the preparation of 2-arylbenzothiazole-6-carboxamides. From 2-(4-chloro-2-hydroxyphenyl)benzothiazole-6-carboxylic acid (0.059 g, 0.19 mmol) and 2-pyrrolidin-1-ylethanamine (0.050 ml, 0.40 mmol), the title compound was isolated (0.018 g, 23%).

APCI-MS m/z: 402.1 [MH⁺].

25

General procedure for the reduction of 2-arylbenzothiazole-6-(amine-1-ylcarbonyl) into 2-arylbenzothiazole-6-(amine-1-ylmethyl)

The 2-arylbenzothiazole-6-(amine-1-ylcarbonyl) (0.2 mmol) was dissolved in 5 ml of THF. After cooling at 0°C, a 1 M solution of LiAlH₄ in THF (0.4 mmol) was added dropwise. The ice-bath was removed and the reaction mixture stirred at rt until completion (LC-MS). Water (0.5 ml) was added followed by CH₂Cl₂ (30 ml). After washing with brine, drying and evaporation, the crude title product was purified by column chromatography (MeOH-CH₂Cl₂, 5:95).

Compound B1 was synthesized following this general procedure (and the general procedures for the synthesis of the corresponding amides). The starting amides were synthesized according to the methods outlined above.

5 *Preparation of Compound B1*

5-chloro-2-[6-(pyrrolidin-1-ylmethyl)-1,3-benzothiazol-2-yl]phenol

From 2-(4-chloro-2-hydroxyphenyl)benzothiazole-6-(pyrrolidin-1-ylcarbonyl) (0.065 g, 0.18 mmol), the title compound was isolated (0.028 g, 45%).

10 ¹H NMR (400 MHz, CD₂Cl₂): δ12.72 (1H, brs); 8.03 (1H, s); 7.97 (1H, d); 7.68 (1H, d); 7.57 (1H, d); 7.13 (1H, d); 6.99 (1H, dd); 3.87 (2H, s); 2.74-2.60 (4H, m); 1.91-1.82 (4H, m).

APCI-MS m/z: 345.1 [MH⁺]

Preparation of Compound C1

15 **6-Phenyl-1H-pyrazolo[4,3-c]cinnolin-3-ol**

A solution of 6-bromo-1H-pyrazolo[4,3-c]cinnolin-3-ol (20 mg, 0.075 mmol), phenyl boronic acid (27 mg, 0.22 mmol, 3.0 equiv.) and {1,1'-Bis(diphenylphosphino)ferrocene}-dichloropalladium(II) (3.1 mg, 5 mol%) in a mixture of dioxane (400 μL), EtOH (40 μL) and aqueous 2M sodium carbonate was heated in a sealed vial to 90°C for 3 hours. After
20 cooling, EtOAc (5mL) was added and the resulting suspension was filtered through a pad of celite. The solid was washed with acetonitrile and 50% aqueous acetonitrile. After evaporation of solvents the remaining crude was dissolved in 50% aqueous acetonitrile, acidified by 2-3 drops of trifluoroacetic acid and purified by reversed phase HPLC. After freeze-drying, the title product was isolated as its trifluoroacetic acid salt as a red powder.

25

¹H NMR (400 MHz, DMSO-*d*₆+D₂O): δ8.06 (1H, brd); 7.80-7.43 (7H, m).

¹H NMR (400 MHz, DMSO-*d*₆+NaOH): δ8.14 (1H, dd); 7.62 (2H, dd); 7.48-7.35 (3H, m); 7.35-7.31 (2H, m).

APCI-MS m/z: 263.1 [MH⁺].

30

Preparation of Compound C7

6-Pyridin-3-yl-1H-pyrazolo[4,3-c]cinnolin-3-amine

A solution of 6-bromo-1H-pyrazolo[4,3-c]cinnolin-3-amine (20 mg, 0.075 mmol), and 3-pyridyl boronic acid (28 mg, 0.22 mmol, 3.0 equiv.) and {1,1'-
35 Bis(diphenylphosphino)ferrocene}-dichloropalladium(II) (3.1 mg, 5 mol%) in a mixture of

dioxane (400 μ L), EtOH (40 μ L) and aqueous 2M sodium carbonate was heated in a sealed vial to 90°C for 3 hours. After cooling, EtOAc (5mL) was added and the resulting suspension was filtered through a pad of celite. The solid was washed with acetonitrile and 50% aqueous acetonitrile. After evaporation of solvents the remaining crude was dissolved in 50% aqueous acetonitrile, acidified by 2-3 drops of trifluoroacetic acid and purified by reversed phase HPLC. After freeze-drying, the title product was isolated as its trifluoroacetic acid salt as a red powder.

¹H NMR (400 MHz, DMSO-*d*₆): δ 13.12 (1H, brs), 8.92 (1H, d), 8.64 (1H, dd), 8.34 (1H, dd), 8.16 (1H, dt), 7.98 (1H, t), 7.92 (1H, d), 7.55 (1H, dd), 6.21 (2H, brs).
APCI-MS *m/z*: 263.1 [MH⁺]

Preparation of Compound D5

6-Bromo-2-(4-isopropylphenyl)-3H-imidazo[4,5-b]pyridine trifluoroacetate

A mixture of 5-bromo-2,3-diaminopyridine (0.060 g, 0.32 mmol) and 4-isopropylbenzaldehyde (0.044 g, 0.30 mmol) and iron(III)chloride hexahydrate (0.48 g, 1.8 mmol) in DMF (200 ml) was heated to 120°C with continuous air bubbling through the solution for 4-16 h until the starting materials has been consumed.
The reaction mixture was poured onto ice/water, filtrated, washed with water, EtOH, MeOH and dried. Recrystallized twice from DMF (250 ml resp.150 ml), the crystals were washed with MeOH, diethyl ether and dried to afford the title compound.

¹H NMR (400 MHz, DMSO-*d*₆): δ 8.40 (1H, brd); 8.25 (1H, brs); 8.15 (2H, brd); 7.46 (2H, brd); 2.98 (1H, m); 1.25 (6H, d).
APCI-MS *m/z*: 316.0/318.0 [MH⁺]

Preparation of Compound D6

6-Bromo-2-(4-isopropoxyphenyl)-3H-imidazo[4,5-b]pyridine trifluoroacetate

A mixture of 5-bromo-2,3-diaminopyridine (0.060 g, 0.32 mmol) and 4-isopropoxybenzaldehyde (0.049 g, 0.30 mmol) and iron(III)chloride hexahydrate (0.48 g, 1.8 mmol) in DMF (200 ml) was heated to 120°C with continuous air bubbling through the solution for 4-16 h until the starting materials has been consumed.
The reaction mixture was poured into icewater, filtrated, washed with water, EtOH, MeOH and dried. Recrystallized twice from DMF (250 ml resp.150 ml), filtered, washed with MeOH, diethyl ether and dried to afford the title compound.

¹H NMR (400 MHz, DMSO-*d*₆): δ8.36 (1H, brs); 8.20 (1H, brs); 8.15 (2H, brd); 7.10 (2H, brd); 4.75 (1H, m); 1.31 (6H, d). APCI-MS *m/z*: 332.0/334.0 [MH⁺]

5

Assays

Itk LANCE TRF assay

The Itk kinase assay utilized recombinant human Itk kinase domain fused with GST (Glutathione S-Transferase). The protein was expressed in High five insect cells, purified in one step on an affinity chromatography glutathione column and stored in 50 mM Tris/HCl (pH 7.6), 150 mM NaCl, 5% (w/v) mannitol, 1 mM DTT, 30% glycerol at -70°C. The kinase substrate used in the assay was a biotinylated peptide derived from the Src-optimal substrate (Nair *et al*, J. Med. Chem., 38: 4276, 1995; biotin-AEEEIYGEFEAKKKK). The assay additions were as follows: Test compounds (or controls; 1 µl in 100% DMSO) were added to black 96-well flat-bottomed plates (Greiner 655076) followed by 20 µl Itk in assay buffer and the reaction was started by adding 20 µl ATP and peptide substrate in assay buffer. The assay buffer constitution during phosphorylation was: 50 mM HEPES (pH 6.8), 10 mM MgCl₂, 0.015% Brij 35, 1 mM DTT, 10% glycerol, 160 ng/well Itk, 2 µM peptide substrate and 50 µM ATP. The assay was stopped after 50 minutes (RT) by adding 150 µl ice-cold Stop solution (50 mM Tris/HCl, pH 7.5, 10 mM EDTA, 0.9% NaCl and 0.1% BSA) together with LANCE reagents (2 nM PT66-Eu³⁺, Wallac AD0069 and 5 µg/mL Streptavidin-APC, Wallac AD0059. Both concentrations were final in stopped assay solution). The plates were measured on a Wallac 1420 Victor 2 instrument with TRF settings after 1h incubation, and the ratio (665 signal/615 signal)*10000 was used to calculate the inhibition values. IC50 values were determined using XLfit.

Btk LANCE TRF assay

The Btk kinase assay utilized recombinant human full length Btk fused with GST (Glutathione S-Transferase). The protein was expressed in baculo virus transfected Sf9 insect cells, purified in one step on an affinity chromatography glutathione-sepharose column and stored in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM DTT and 30 % glycerol and stored at -70°C. The kinase substrate used in the assay was Btk217-229

(biotin-KKVVALYDYMPMN) comprising the Btk autophosphorylation site. The assay additions were as follows: Test compounds (or controls; 1 μ l in 100% DMSO) were added to black 96-well flat-bottomed plates (Greiner 655076) followed by 20 μ l Btk and substrate in assay buffer and the reaction was started by adding 20 μ l ATP. The assay buffer constitution during phosphorylation was: 50 μ M Tris/HCl (pH 7.4), 7.5 mM MnCl₂, 0.002% Brij 35, 10 mM DTT, 5% glycerol, 40 ng/well Btk, 2 μ M peptide substrate and 20 μ M ATP. The assay was stopped after 60 minutes (RT) by adding 120 μ l ice-cold Stop solution (50 mM Tris/HCl, pH 7.5, 10 mM EDTA, 0.9% NaCl and 0.1% BSA) together with LANCE reagents (1 nM PT66-Eu³⁺, Wallac AD0069 and 10 nM Streptavidin-APC, Wallac AD0059. Both concentrations were final in stopped assay solution). The plates were measured on a Wallac 1420 Victor 2 instrument with TRF settings after 1h incubation, and the ratio (665 signal/615 signal)*10000 was used to calculate the inhibition values. IC₅₀ values were determined using XLfit.

15 *Lyn LANCE TRF assay*

The Lyn kinase assay utilized recombinant N-terminal 6His-tagged human Lyn. The protein was expressed in baculo virus infected Sf9 insect cells and used as a crude cell lysate, stored at -70°C. The autophosphorylation site Lyn 363-369 (biotin-EDNEYTA) from human p56 Lyn was used as kinase substrate in the assay. The assay additions were as follows: Test compounds (or controls; 1 μ l in 100% DMSO) were added to black 96-well flat-bottomed plates (Greiner 655076) followed by 20 μ l Lyn in assay buffer and the reaction was started by adding 20 μ l ATP/substrate. The assay buffer constitution during phosphorylation was: 90 mM Hepes (pH 7.4), 1.5 mM MnCl₂, 0.09% Triton X-100, 80 μ M DTT, 80 μ M Na₃VO₄, Lyn lysate diluted 1/20000, 7 μ M peptide substrate and 7.5 μ M ATP. The assay was stopped after 100 minutes (RT) by adding 120 μ l ice-cold Stop solution (100 mM Hepes, pH 7.4, 0.1% Triton X-100 and 10 mM EDTA) together with LANCE reagents (2 nM PT66-Eu³⁺, Wallac AD0069 and 116 nM Phycolink Streptavidin-APC, Prozyme PJ25S. Both concentrations were final in stopped assay solution). The plates were measured on a Wallac 1420 Victor 2 instrument with TRF settings after 1h incubation, and the ratio (665 signal/615 signal)*10000 was used to calculate the inhibition values. IC₅₀ values were determined using XLfit.

Syk assay

Syk kinase was cloned in baculovirus and expressed as a full-length protein in Sf9 cells (AZAP). A crude enzyme extract was used as enzyme source. As substrate SLP76(107-120) (Biotin-6-aminohexanoate-Ser-Phe-Glu-Glu-Asp-Asp-Tyr-Glu-Ser-Pro-Asn-Asp-Asp-Gln-OH) synthesised in house was used. The reaction was performed in flat bottom 384-well solid black plates (Costar) in a final volume of 20 µl, containing 50 mM Hepes pH 7.4, 0.05% Triton X-100, 0.2 mM DTT, 1 µM ATP, 3 mM MnCl₂, 0.2 mM peptide substrate and a 5000-fold dilution of SYK insect cell extract. After 30 min incubation at room temperature the reaction was terminated by addition of 15 µl EDTA to a final concentration of 20 mM diluted in detection buffer (50 mM Hepes pH 7.4, 0.17% BSA, 0.033% Tx-100, 250 mM NaCl). For detection 15 µl of PT66-acceptor and SA-donor beads in detection buffer were added to a final concentration of 15 µg/ml. The plates were sealed and incubated in the dark for 2-3 hours at room temperature under constant agitation before measurement in the AlphaQuest™ AD microplate analyser.

15

Basophil Histamine Release assay:

Basophils were obtained from human blood by density centrifugation on polymorphprep (AXIS-SHIELD). Mononuclear cells were collected from the top layer and washed in THG⁻ buffer (0.8% NaCl, 0.24% HEPES, 0.1% D-glucose anhydrous, 0.1% Gelatine Type B, 0.1% 1.8M NaH₂PO₄ · 2 H₂O, 0.1% 2.7M KCl/ 1 L H₂O). Basophils were purified by negative selection using MACS Basophil Isolation Kit (Miltenyi Biotech). Briefly, cells were labelled with magnetic beads in cold MACS buffer (PBS, 2 mM EDTA, and 0.5% BSA) and separated using the AutoMACS separation instrument. The cells were subsequently washed in RPMI 1640 supplemented with 25 mM HEPES (GIBCO-BRL) and 5% heat inactivated Fetal Calf Serum (GIBCO-BRL), and incubated with 5 µg IgE (Serotec PHP008) for 60 minutes at 37°C. The cells were washed in THG⁻ buffer and resuspended in THG⁺ buffer (THG⁻, supplemented with 1.8 mM CaCl₂ · 2 H₂O and 1 mM MgCl · 6 H₂O). The basophils (1.5 x 10⁴ /well) were added to round bottomed 96-well polypropylene plates (Costar 3365) in THG⁺ buffer and stimulated through FcεRI cross linking by addition of 10 ng anti-IgE/well (The Binding Site). Plates were sealed with plate sealers (LabSystem) and incubated at 37°C in a water bath for 25 minutes. The release was stopped by centrifugation at 4°C and supernatants collected.

Histamine content was measured in the supernatants by addition of 20 µl HMT/³H-SAM [1 ml Histamine Methyl Transferase (HMT), purified from male rat kidneys (for description see below) and 1 Mbq S-Adenosyl-L-[methyl-³H] methionine (Amersham

35

Pharmacia Biotech) in 5 ml THG⁺⁺ buffer] to 100 µl sample (volume adjusted with THG⁺⁺ buffer). For total histamine release 1.5×10^4 boiled basophils were used. Samples were incubated at 37°C for 60 minutes and the reaction stopped by addition of 50 µl NaOH (10 mM) followed by 400 µl toluene/isoamyl alcohol (4:1). Tubes were capped and vigorously
5 shaken for 40 sec. to extract methyl histamine and subsequently centrifuged to separate the phases. To measure ³H-methyl histamine content in the top (organic) phase, samples were transferred to LumaPlates (Packard), dried and read in 1450 MicroBeta instrument (Wallac).

HMT – Histamine Methyl Transferase preparation:

10 HMT was obtained from male rat kidneys. Briefly, the membrane was peeled off and kidneys (80 g) homogenized with 10 volumes of 0.25 M sucrose. The homogenate was centrifuged at 15000xg for 90 minutes at 0°C. The supernatant was collected and saturated to 45% with ammonium sulphate (277 mg/ ml) whilst stirring in the cold and subsequently centrifuged at 10000g for 20 min. The supernatant was collected, adjusted
15 to 70% saturation with ammonium sulphate (171 mg/ ml) and centrifuged at 10000g for 15 min. Supernatants were discarded and pellets dissolved in 0.1 M sodium phosphate buffer pH 7.4, and dialysed against 2 x 4 L of cold 1 mM sodium phosphate buffer pH 7.4.

Basophil Leukotriene C4 (LTC4) Release assay

20 Human basophils (purified according to the protocol described for basophil histamine release assay above) were resuspended to a density of 600,000/ ml in THG⁻ buffer. Cells (50 µl) were added to 96 well plates containing 25 µl compound (4x final concentration i.e. 4×10^{-9} to 4×10^{-6} M). Release was started by the addition of 10 µl of 10 times concentrated anti-human IgE to give a final dilution of 1:10000 (Sigma code I-0632) at
25 37°C for 25 minutes. Cells were spun at 300xg for 5 minutes at 4°C and 50 µl supernatant sampled for the measurement of LTC4.

LTC4 was measured by a commercial ELISA kit (BioTRAK™ Leukotriene C4/D4/E4 Enzyme immunoassay system RPN224). Assays were carried out as per kit instructions. Briefly, 50 µl supernatant from basophil release experiments were sampled into the
30 biotrak ELISA plate, 50 µl of anti-serum was added and incubated for 2 hours at room temperature. Subsequently 50 µl of peroxidase conjugate was then added and incubated for a further hour at room temperature. Wells were then emptied and washed four times

with 300 µl wash buffer. Substrate (150 µl) was then added to each well and incubated for 30 minutes on a plate shaker at room temperature. Sulphuric acid (100 µl) was then added and the OD determined at 450 nm. LTC₄ released was calculated by comparison to a standard curve set up as in the kit, but using release buffer (THG). Briefly, serial
5 dilutions of LTC₄ were made in THG and 50 µl of these transferred to the Biotrak plate to give standards of 0, 0.75, 1.5, 3, 6, 12, 24, and 48 pg of LTC₄/well.

Basophil Interleukin-4 (IL-4) Release assay

Human basophils (purified according to the protocol described for basophil histamine
10 release assay above) were resuspended to a density of 2×10^6 /ml in HEPES-buffered RPMI 1640 with 10 % FCS and 2 mM L-glutamine. Cells (50 µl) were added to 96 well plates containing 25 µl compound (4x final concentration i.e. 4×10^{-9} to 4×10^{-6} M). Release was started by the addition of 10 µl of 10 times concentrated anti-human IgE to give a final dilution of 1:10,000 (Sigma code I-0632) at 37°C for 4 hours. Cells were spun
15 at 300xg for 5 minutes at 4°C and 50 µl supernatant sampled for the measurement of IL-4. IL-4 was measured by a commercial ELISA kit (BioTRAK™, Interleukin-4 [(h)IL-4] Human, Elisa System, code RPN2753). Assays were carried out as per kit instructions. Briefly, 50 µl of biotinylated antibody reagent was added to the BioTRAK™ plate. Supernatant from basophil release experiments were sampled into the plate. This was
20 then incubated at room temperature for two hours at room temperature. Wells were then emptied and washed three times with 300 µl wash buffer. Streptavidin-HRP conjugate (100 µL) was then added and incubated at room temperature for 30 minutes. Wells were then emptied and washed three times with wash buffer. Substrate (100 µl) was then added to each well and incubated for 30 minutes in the dark at room temperature. Stop
25 solution (100 µl 0.18 M sulphuric acid) was then added and the OD determined at 450nm. IL-4 released was calculated by comparison to a standard curve set up as in the kit, but using release buffer (RPMI 1640). Briefly, serial dilutions of IL-4 were made and 50 µl of these transferred to the Biotrak plate to give standards of 0, 0.08, 0.2, 0.51, 1.28, 3.2, 8, and 20 pg of IL-4/well.

Lung Mast Cell Histamine Release assay

Human lung tissue was obtained from lung resection patients. The tissue (usually 5-20 g) was chopped on arrival with scissors into strips of approximately 5 mm by 20 mm, and stored overnight in 50ml RPMI 1640 with 5% FCS and Penicillin streptomycin solution (Gibco, 10,000 µg /mL of Streptomycin sulphate and 10,000 Units/mL of penicillin G) 1 ml/100 ml RPMI. The following day the tissue was finely chopped using a mechanical tissue chopper (McIlwain tissue chopper™) into small cubes (~1 mm³) and washed through nylon gauze with 1L THG (Tyrodes-HEPES-gelatin pH 7.4). The washed tissue was then suspended in 10X volume (w/v) of digestion buffer (THG containing 1 mM MgCl₂, 5 mg/ml bovine serum albumin, 0.01 mg/ml DNase 1 type IV (Sigma), and 1 mg/ml collagenase type 1A (Sigma)) and incubated for 30 minutes at 37°C. Tissue was further disrupted by passing through a 50 ml syringe (with tip removed) 10 times, and incubated for a further 30 minutes in the digestion buffer. The digestion buffer was then diluted 1:1 with THG. Isolated cells were harvested by passing through 120 µm nylon gauze and centrifugation at 300xg for 5 minutes at 20°C. The cells were washed 3 x in THG. After the final wash the cells were resuspended in 50 ml RPMI-1640 containing 5% FCS 1% glutamine and 1% Penicillin /streptomycin and stored overnight at 4°C. Mast cells were purified by positive magnetic staining using a PAN mouse IgG1 CELLection™ kit (Dyna) Beads (300 µl) were incubated overnight at 4°C with 50 µl of anti-human CD117 (Pharmingen). The beads were then washed 4 X in THG and collected using a Dynal magnet. The prepared cells were then centrifuged at 300g for 5 minutes at 4°C and resuspended in 2 mL of THG and pre-treated Dynal beads added (10 beads/mast cell). The cells and beads were mixed and left for 1 hour on a roller mixer at 4°C. At the end of this incubation the cells were diluted with a further 8 ml of THG and cells attached to beads were isolated using the Dynal magnet, and washed 5 x in THG (10 mls) . The beads were then removed from the cells by incubation with 200 µl THG containing 20 µl of releasing buffer (supplied with Dynal CELLection™ kit) for 15 minutes at 37°C. Cells were aspirated in a pipette 5 times and detached beads removed using the Dynal magnet. Purified mast cells were collected in the supernatant. The beads were washed in THG (200 µl) a further 3 times to maximise recovery of mast cells. The supernatants were

pooled and centrifuged at 300xg 5 minutes at room temperature. The cells were then washed in 10 ml THG and resuspended into 1 ml RPMI-1640 with 5%FCS. Mast cells were counted in a haemocytometer (20 μ l cells + 180 μ l Kimura's stain).

Pure mast cells (1 ml in RPMI 1640 containing 5% FCS) were sensitised by incubating
5 with 5 μ g/ml human IgE (Serotec) for 1 hour at 37°C. Cells were then diluted to 10 ml with THG and centrifuged, 300xg for 5 minutes at room temperature. The pellet was washed in 10 ml THG⁻ and cells recovered by centrifugation, 300xg for 5 minutes at room temperature. The pellet was resuspended in 1 ml THG⁺⁺. 10 μ l of the cells were then taken into 90 μ l Kimura's stain and counted for purity and number in a haemocytometer.
10 Mast cells were then resuspended to a density of 300,000 mast cells/ml in THG⁺⁺ for use in release experiments.

Stock compounds at 4 mM were made up in DMSO and serially diluted in DMSO to 4000X final concentration. Each of these serial dilutions was then diluted 1000X into THG⁺⁺. These solutions were then used at 1/4 final volume in release wells (25 μ l
15 compound + 50 μ l cells, 15 μ l buffer and 10 μ l anti-IgE (Sigma)).
50 μ l cells were added to wells of a 96 well plate (15,000 cells/well) and compound or vehicle added (25 μ l). The cells were pre-warmed to 37°C and left for 3 minutes with compound before activation with 10 μ l of 10x concentrated anti-human IgE (1/3,000 final anti-IgE (sigma I-0632)) in THG⁺⁺ buffer. Release was allowed to continue for 20 minutes
20 at 37°C. Plates were spun at 200xg for 5 minutes at 4°C and 75 μ l supernatant sampled into 1 ml deep well plates for analysis of histamine.

Total histamine was determined by freeze-thawing 50 μ l of mast cells with 150 μ l distilled water and microwaving for 30 seconds. 25 μ l of this is sampled for Histamine analysis. Histamine content was determined according to the same protocol as for the basophil
25 histamine release assay described above.

EXAMPLE 1

The structurally diverse compounds A5, A14, B1, B3, C1, C7, D5 and D6 were tested for
30 their ability to inhibit histamine release from primary human basophils isolated from blood obtained from healthy volunteers. The basophils were stimulated with anti-IgE.

The compounds were also profiled to determine their capability to inhibit the catalytic activity of the protein tyrosine kinases Btk, Syk, and Lyn which are all present in mast cells and important in the signalling downstream of the FcεRI receptor.

5

Results are shown in the table below.

Compound	Itk IC ₅₀ (μM)	Btk IC ₅₀ (μM)	Lyn IC ₅₀ (μM)	Syk IC ₅₀ (μM)	algE induced histamine IC ₅₀ (μM)
A5	0.05	>50	43.8	4.57	0.51
A14	0.252		>50	8.82	0.99
B1	0.43	>50	>50	>50	0.18
B3	0.978	>50	>50	>50	0.20
C1	0.534	0.276	4.32	>50	0.10
C7	6.73	0.139	7.18	>50	10
D5	0.264	>50	>50	5.94	0.38
D6	0.316	>50	>50	45.69	0.35

Results show that inhibition of anti-IgE induced histamine release was dependent on Itk inhibition.

10

Compound C7 is a selective Btk inhibitor which does not have the preferred Itk inhibitory activity (Itk IC₅₀ is not less than 5 μM). Compound C7 does *not* inhibit histamine release. Each of the other compounds is a good Itk inhibitor (Itk IC₅₀ less than 5 μM) and each inhibits histamine release. Compounds A5, B1, B3, D5 and D6 are selective Itk inhibitors (Btk IC₅₀ >50 μM compared to Itk IC₅₀ <5 μM). Compounds A5, A14, B1, B3, D5 and D6 are more than 10-fold selective over the kinases Syk and Lyn. Compound C1 is a non-selective Itk inhibitor which is more active against Btk.

15

EXAMPLE 2

Two of the compounds tested in Example 1 were tested for their ability to inhibit IL-4 and/or LTC₄ release from anti-IgE stimulated human primary basophils. Results are shown in the table below. Note that the IL-4 release was measured in the presence of 10% serum, which reduces the effect of the compound due to compound binding to the serum proteins.

Compounds A5 and C1 inhibited LTC₄ release. Compound A5 also inhibited IL-4 release. Compound C1 was not tested in the IL-4 assay.

Compound	Itk IC ₅₀ (μ M)	algE induced LTC ₄ release prim. basophils IC ₅₀ (μ M)	algE induced IL-4 release prim. basophils IC ₅₀ (μ M)
A5	0.05	0.20	1.2
C1	0.53	0.26	

10

EXAMPLE 3

Two of the compounds tested in Example 1 were tested for their ability to inhibit histamine release from human lung mast cells prepared from human lung tissue. These compounds were found to inhibit histamine release from primary human lung mast cells (results shown in the table below).

Compound	Itk IC ₅₀ (μ M)	algE indu. histamine prim. basophils IC ₅₀ (μ M)	algE indu. histamine lung mast cells IC ₅₀ (μ M)
D5	0.264	0.38	0.2
C1	0.534	0.10	0.2

20

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CLAIMS

What we claim is:

- 5 1) A method of treating or preventing a mast cell-driven or a basophil-driven condition or disease which comprises administering a therapeutically effective amount of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof to a human or mammal suffering from or susceptible to the condition or disease, wherein the Itk inhibitor gives an IC_{50} value for inhibition of Itk activity *in vitro* of less than 5 μ M.
- 10 2) A method as claimed in claim 1 for treating or preventing a mast cell-driven condition or disease wherein the Itk inhibitor gives an IC_{50} value for inhibition of Btk activity *in vitro* which is more than 50-fold greater than its IC_{50} value for inhibition of Itk activity *in vitro*.
- 15 3) A method as claimed in either claim 1 or claim 2 for treating or preventing a reversible obstructive airway disease.
- 4) A method as claimed in claim 3 for treating or preventing asthma.
- 20 5) A method as claimed in either claim 1 or claim 2 for treating or preventing rhinitis
- 6) A method as claimed in either claim 1 or claim 2 for treating or preventing chronic obstructive pulmonary disease.
- 25 7) Use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof for the treatment or prevention of a mast cell-driven or a basophil-driven condition or disease, wherein the Itk inhibitor gives an IC_{50} value for inhibition of Itk activity *in vitro* of less than 5 μ M.
- 30 8) Use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof as claimed in claim 7 wherein the Itk inhibitor gives an IC_{50} value for inhibition of Btk activity *in vitro* which is more than 50-fold greater than its IC_{50} value for inhibition of Itk activity *in vitro*.
- 35

- 9) Use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof as claimed in either claim 7 or claim 8 for the treatment or prevention of a reversible obstructive airway disease.
- 5 10) Use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof as claimed in claim 9 for the treatment or prevention of asthma.
- 11) Use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof as claimed in either claim 7 or claim 8 for the treatment or prevention of rhinitis.
- 10 12) Use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof as claimed in either claim 7 or claim 8 for the treatment or prevention of chronic obstructive pulmonary disease.
- 13) Use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof in the
15 manufacture of a medicament for the treatment or prevention of a mast cell-driven or a basophil-driven condition or disease, wherein the Itk inhibitor gives an IC_{50} value for inhibition of Itk activity *in vitro* of less than 5 μ M.
- 14) Use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof as
20 claimed in claim 13 wherein the Itk inhibitor gives an IC_{50} value for inhibition of Btk activity *in vitro* which is more than 50-fold greater than its IC_{50} value for inhibition of Itk activity *in vitro*.
- 15) Use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof as
25 claimed in either claim 13 or claim 14 in the manufacture of a medicament for the treatment or prevention of a reversible obstructive airway disease.
- 16) Use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof as
30 claimed in claim 15 in the manufacture of a medicament for the treatment or prevention of asthma.
- 17) Use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof as claimed in either claim 13 or claim 14 in the manufacture of a medicament for the treatment or prevention of rhinitis.

- 18) Use of an Itk inhibitor or a pharmaceutically acceptable salt or ester thereof as claimed in either claim 13 or claim 14 in the manufacture of a medicament for the treatment or prevention of chronic obstructive pulmonary disease

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 03/01274

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61K 31/502, A61P 11/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI DATA, CHEM.ABS.DATA, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 0250071 A1 (BRISTOL-MYERS SQUIBB COMPANY), 27 June 2002 (27.06.02), claims 21-22 --	1-18
X	WO 0125220 A1 (KINETIX PHARMACEUTICALS INC.), 12 April 2001 (12.04.01), page 31, line 6; page 33, line 27 - line 34 --	1-18
A	WO 0147922 A2 (AVENTIS PHARMA LIMITED), 5 July 2001 (05.07.01) --	1-18
A	WO 9924035 A1 (BRISTOL-MYERS SQUIBB COMPANY), 20 May 1999 (20.05.99) -- -----	1-18

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

17 November 2003

Date of mailing of the international search report

19 -11- 2003

Name and mailing address of the ISA/

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE03/01274

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **1-12**
because they relate to subject matter not required to be searched by this Authority, namely:
see next sheet
2. ☒ Claims Nos.: **1-18**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see next sheet
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

national application No.
PCT/SE03/01274

Box. 1.1

Claims 1-12 relate to methods of treatment of the human or animal body by surgery or by therapy/diagnostic methods practised on the human or animal body/Rule. 39.1.(iv)). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds/compositions.

Box I.2

Present claims 1-18 relate to a large number of possible compounds since the claims cover all Itk inhibitors irrespective of their structures.

The formulation "mast cell-driven or a basophil-driven condition or disease" in claims 1-2, 7 and 13 covers a very large number of diseases. Support within the meaning of Article 6 PCT is to be found, however, for only a very small proportion of the compounds and diseases claimed.

The claims relate also to compounds defined by reference to the following parameter: An IC_{50} value for inhibition of Itk activity in vitro of less than 5 μ M.

The use of these parameters in the present context is considered to lead to a lack of clarity within the meaning of Article 6 PCT. It is impossible to compare the parameters the applicant has chosen to employ with what is set out in the prior art.

In the present case, the claims lack support and clarity, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

Consequently, the search has been focused on the expression "Itk inhibitor" and the examples. The searched diseases have been restricted to airways diseases, asthma and rhinitis.

INTERNATIONAL SEARCH REPORT

Information on patent family members

06/09/03

International application No.

PCT/SE 03/01274

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
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				US	2003069238 A	10/04/03
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				CA	2395593 A	05/07/01
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				HU	0203895 A	28/02/03
				JP	2003519144 T	17/06/03
				NO	20023032 A	21/06/02
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