A selective novel low-molecular-weight inhibitor of I κ B kinase- β (IKK- β) prevents pulmonary inflammation and shows broad anti-inflammatory activity

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1 Pulmonary inflammatory diseases such as asthma are characterized by chronic, cell-mediated inflammation of the bronchial mucosa.

2 Recruitment and activation of inflammatory cells is orchestrated by a variety of mediators such as cytokines, chemokines, or adhesion molecules, the expression of which is regulated *via* the transcription factor nuclear factor kappa B (NF- κ B).

3 NF- κ B signaling is controlled by the inhibitor of kappa B kinase complex (IKK), a critical catalytic subunit of which is IKK- β .

4 We identified COMPOUND A as a small-molecule, ATP-competitive inhibitor selectively targeting IKK- β kinase activity with a K_i value of 2 nM.

5 COMPOUND A inhibited stress-induced NF- κ B transactivation, chemokine-, cytokine-, and adhesion molecule expression, and T- and B-cell proliferation.

6 COMPOUND A is orally bioavailable and inhibited the release of LPS-induced TNF- α in rodents.

7 In mice COMPOUND A inhibited cockroach allergen-induced airway inflammation and hyperreactivity and efficiently abrogated leukocyte trafficking induced by carrageenan in mice or by ovalbumin in a rat model of airway inflammation.

8 COMPOUND A was well tolerated by rodents over 3 weeks without affecting weight gain.

9 Furthermore, in mice COMPOUND A suppressed edema formation in response to arachidonic acid, phorbol ester, or edema induced by delayed-type hypersensitivity.

10 These data suggest that IKK- β inhibitors offer an effective therapeutic approach for inhibiting chronic pulmonary inflammation.

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Abbreviations: A549, human alveolar type II epithelium-like lung adenocarcinoma; AA, arachidonic acid; AHR, airway hyperreactivity; AP-1, activator protein-1; AUC, area under the curve; BAL, bronchoalveolar; CL, blood clearance; ConA, Concanavalin A; DC, dendritic cell; DMSO, dimethyl sulfoxide; DNFB, dinitrofluorobenzene; DTH, delayed-type hypersensitivity; GC(s), glucocorticoid(s); GM-CSF, granulocyte-macrophage colony-stimulating factor; GR, glucocorticoid receptor; GST, glutathione S-transferase; HPF, high-powered field; ICAM, intercellular adhesion molecule; IFN, interferon; IKK, inhibitor of kappa B kinase complex; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; LTB, leukotriene B; MCP, monocyte chemoattractant protein; MED, minimal effective dose; MIP, macrophage inflammatory protein; MKK, mitogen-activated protein kinase kinase; NF- κ B, nuclear factor kappa B; OVA, ovalbumin; PBMC, peripheral blood mononuclear cell; PGE, prostaglandin E; PMA, phorbol myristate acetate; PMSF, phenylmethyl sulfonylfluoride; RANTES, regulated on activation normal T-cell expressed and secreted; SIRS, systemic inflammatory response syndrome; TCR, T-cell receptor; TNF- α , tumor necrosis factor α ; VCAM, vascular cell adhesion molecule

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Introduction

Asthma is a chronic inflammatory disease with increasing incidence worldwide (Beasley, 2002). While mild to moderate asthma is well controlled by inhaled glucocorticoids (GCs) and β_2 -agonists, about 5% of patients do not well respond to this treatment. Despite this small percentage, these patients contribute disproportionately high (about 50%) to the overall health-care cost of asthma (Adcock & Ito, 2004). Little is known about the underlying pathology of steroid-resistant asthma, but it seems that one-half to two-thirds of severe asthmatics show persistent large airway tissue eosinophilia and other signs of inflammation despite continued high-dose steroid treatment (Wenzel, 2003). On a molecular level, it is still unclear how exactly GCs exert their anti-inflammatory activity and, consequently, steroid resistance is not well understood. Recent evidence indicates that a major part of the anti-inflammatory effects of GCs is based on transrepressing and therefore inhibiting the proinflammatory transcription factors activator protein-1 (AP-1) or nuclear factor kappa B (NF- κ B). One hypothesis for steroid insensitivity is increased activation/expression of AP-1 or NF- κ B proteins which cannot be sufficiently transrepressed by GC-bound glucocorticoid receptor (GR) (De Bosscher *et al.*, 2003; Leung & Bloom, 2003). Alternative hypotheses include the reduced ability of the GR to bind to the ligand or increased expression of an alternatively spliced, dominant-negative GR (GR- β). The first hypothesis is supported by a recent study showing that persistent activation of NF- κ B signaling is observed in severe, uncontrolled asthma (Gagliardo *et al.*, 2003). In addition, a variety of data both from asthma patients as well as from deletion mutants of NF- κ B proteins in mice support a critical role for NF- κ B in asthma pathology (Yang *et al.*, 1998; Donovan *et al.*, 1999; Christman *et al.*, 2000). Inhibition of NF- κ B activity might therefore be an effective alternative approach to treat asthma, including severe forms refractory to GCs.

NF- κ B proteins are a family of ubiquitously expressed transcription factors that, in mammals, consist of five members: p65 (RelA), RelB, c-Rel, NF- κ B1 (p50 and its precursor 105) and NF- κ B2 (p52 and its precursor p100) (Verma *et al.*, 1995). NF- κ B and related family members are involved in the regulation of more than 50 genes, which are activated upon inflammatory and immune responses (Baeuerle & Baichwal, 1997). NF- κ B shows a unique mode of regulation: It is kept in an inactive state in the cytoplasm by interacting with members of the I κ B family of proteins which mask the nuclear translocation signal of NF- κ B. Upon stimulation of cells by various cytokines (e.g. TNF- α , IL-1 β), CD40 ligand, lipopolysaccharide (LPS), oxidants, mitogens (e.g. phorbol ester) or viruses, I κ B proteins become phosphorylated at specific serine residues by the inhibitor of κ B (IKK) kinase complex. This triggers poly-ubiquitinylation and subsequent degradation through a proteasome-dependent pathway, resulting in transcriptionally active NF- κ B (Verma *et al.*, 1995; Baeuerle & Baichwal, 1997; Rothwarf & Karin, 1999; Gosh & Karin, 2002; Yamamoto & Gaynor, 2004). IKK activity resides in a high-molecular-weight complex comprising of at least two catalytic subunits, IKK- α (IKK1) and IKK- β (IKK2), and the associated regulatory subunit IKK- γ /NEMO (Rothwarf & Karin, 1999; Gosh & Karin, 2002; Yamamoto & Gaynor, 2004). Although IKK- α and IKK- β have a high degree of sequence homology and share similar structural

domains, IKK- β has a 20–50-fold higher level of kinase activity for I κ B than does IKK- α (Li *et al.*, 1998). More dramatic differences became obvious by the generation of IKK- α - and IKK- β -deficient mice. *ikk- α ^{-/-}* mice presented an unexpected phenotype including shorter limbs and skull, and a fused tail, all enveloped in a shiny and sticky skin (Hu *et al.*, 1999; 2001; Takeda *et al.*, 1999; Sil *et al.*, 2004). These mice die perinatally and have hyperproliferative epidermal cells that do not differentiate. However, IL-1 β - and TNF- α -induced NF- κ B activation is normal, and the phosphorylation and degradation of I κ B proteins is also unchanged. Thus, IKK- α is involved in dermal and skeletal development and cannot be compensated for by IKK- β . Furthermore, it was recently shown that IKK- α plays a role in B-cell maturation and secondary lymphoid organ formation through processing of the NF- κ B2 precursor p100 (Senftleben *et al.*, 2001; Muller & Siebenlist, 2003). On the other hand, IKK- β -deficient mice (*ikk- β ^{-/-}*) die as embryos and show massive liver degeneration due to hepatocyte apoptosis (Li QT *et al.*, 1999; Li ZW *et al.*, 1999; Tanaka *et al.*, 1999). In these mice, marked defects in the activation of the NF- κ B pathway triggered by pro-inflammatory cytokines such as TNF- α became obvious (Tanaka *et al.*, 1999).

Although IKK- α and - γ have also been shown to be involved in NF- κ B stimulation by cytokines (Rudolph *et al.*, 2000; Li *et al.*, 2002), it is generally believed that during inflammation IKK- β is more critical than IKK- α in activating the NF- κ B pathway and almost all proinflammatory functions reported for NF- κ B require the IKK- β subunit for activation. Thus, we aimed at identifying a small-molecule inhibitor selectively targeting IKK- β . This study characterizes the biochemical, cellular and *in vivo* anti-inflammatory profile of COMPOUND A, a potent and selective inhibitor of IKK- β . Our results highlight the therapeutic potential of such compound for the treatment of asthma and other inflammatory diseases.

Methods

Kinase assays using recombinant proteins

IKK assay Human IKK- α and IKK- β were cloned from Quickclone cDNA library (BD Biosciences Clontech, Palo Alto, CA, U.S.A.) by polymerase chain reaction. The IKK- β kinase assay was performed as described (Murata *et al.*, 2003).

For the IKK- α kinase assay, enzyme, substrate, and assay buffer were replaced by recombinant IKK- α (final concentration 1.5 μ g ml⁻¹), ATP (final concentration 0.2 μ M ATP, 0.5 μ Ci well⁻¹ [γ -³³P]ATP), GST-I κ B α full length (final concentration 0.2 μ g ml⁻¹), and 20 mM HEPES, pH 7.6, 20 mM β -glycerophosphate, 1 mM Na₃VO₄, 10 mM MgCl₂, 2 mM MnCl₂, 100 mM KCl, 1 mM DTT, 0.4 mM PMSF, 0.1 mg ml⁻¹ BSA, respectively.

Other enzymes were tested according to published procedures or results were obtained from MDS pharmacology services (<http://www.mdsps.com/>).

Immune complex kinase assay

Endogenous IKK complexes were immunoprecipitated from lysates of A549 cells. Subconfluent cells were left untreated or

stimulated with the indicated concentrations of recombinant human TNF- α (R&D Systems, Oxford, U.K.) for 15 min. The cells were washed with cold PBS twice and lysed in lysis buffer, 50 mM HEPES, pH 7.6, 10% glycerol, 1 mM sodium metabisulfite, 1 mM NaF, 20 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM benzamidine hydrochloride, 20 mM *p*-nitrophenylphosphate, 100 mM NaCl, 1% NP-40, 1 mM EDTA and complete proteinase inhibitor mix. Insoluble debris was removed by centrifugation and 100 μ g of cell lysate was incubated with 2 μ g anti-IKK- α antibody (H-744, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) in 100 μ l lysis buffer supplemented with 250 mM NaCl. After incubation for 2 h on ice, 20 μ l protein A beads (50% v v⁻¹) were added, and the mixture was incubated under rotation for an additional 1 h at 4°C. The immunoprecipitates were then washed five times with lysis buffer and subjected to kinase assay or immunoblot analysis.

Immunoprecipitates from 100 μ g of cell lysate were used for kinase assays. The reaction mixture consisted of kinase buffer (20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 20 mM β -glycerophosphate, 1 mM sodium orthovanadate, 20 mM MgCl₂, 0.4 mM PMSF, 2 mM DTT, 20 mM creatine phosphate, 20 mM *p*-nitrophenylphosphate), 3 μ g GST-IkB α (1–54), 5 μ M ATP, and 2.5 μ Ci [γ -³²P]ATP (Amersham Biosciences, Buckinghamshire, England) in a volume of 30 μ l. For compound evaluation, immunoprecipitates were pretreated with COMPOUND A on ice for 30 min at the concentrations indicated. Kinase reactions were performed at 37°C for 30 min, then the reaction mixtures were subjected to SDS-PAGE and autoradiography.

Intracellular phosphorylation

Detection of IKK- α and IKK- β A549 cells were lysed in lysis buffer and 300 μ g lysate was subjected to immunoprecipitation using 0.2, 1, or 2 μ g anti-IKK- α antibody (H-744). In all, 50 μ g of lysate, supernatant, or immunoprecipitated proteins from 100 μ g of lysate were subjected to SDS-PAGE and immunoblot analysis. Blots were developed with anti-IKK- α (H-744) or anti-IKK- β (H-470, Santa Cruz Biotechnology) and horseradish peroxidase-conjugated goat anti-rabbit-IgG (Tago Immunologicals, Camerillo, CA, U.S.A.). Proteins were visualized using chemiluminescence (ECL Plus Western Blotting Detection Reagents, Amersham Biosciences, Buckinghamshire, England).

Detection of IkB α and phosphorylated IkB α Subconfluent A549 cells growing in six-well plates were left untreated or treated with COMPOUND A for 30 min. Then, cells were left untreated or stimulated with the indicated concentration of TNF- α for the indicated period of time. Cells were washed with cold PBS twice and lysed by 100 μ l SDS-PAGE sample buffer on ice. Cell lysates were briefly sonicated, centrifuged, and supernatants were subjected to SDS-PAGE and immunoblot analysis by using anti-IkB α (Cell Signaling Technology, Beverly, MA, U.S.A.) or antiphosphorylated-IkB α antibodies (Cell Signaling Technology, Beverly, MA, U.S.A.) according to the manufacturer's recommendations.

Cell culture and cellular assays

In vitro induction of RANTES by TNF- α in A549 cells was performed as described (Murata *et al.*, 2003). Activated mouse

peritoneal cells were induced by injection of 2 ml of 10% thioglycollate into the peritoneal cavity of BALB/c mice. After 4 days, peritoneal cells were harvested by lavage of the peritoneal cavity with cold PBS. The cells were washed once in RPMI 1640 medium/10% FCS, seeded on culture plates and left to adhere for 1 h before LPS stimulation (see below). Humans dendritic cells (DCs) were generated from PBMC as described with minor modifications (Sallusto & Lanzavecchia, 1994). Briefly, PBMC were isolated from heparinized blood from healthy donors by standard density-gradient centrifugation on 80% Percoll (Amersham Biosciences, Uppsala, Sweden) diluted in PBS. PBMC were washed twice in PBS, resuspended in RPMI-1640 medium (Nacalai Tesque) supplemented with 10% heat-inactivated fetal calf serum (JRH Biosciences, Lenexa, KS, U.S.A.), 292 μ g ml⁻¹ L-glutamine (Invitrogen Corp., Carlsbad, CA, U.S.A.), 100 IU ml⁻¹ penicillin, and 100 μ g ml⁻¹ streptomycin, and monocytes were allowed to adhere on 24-well plates for 60 min. Nonadherent cells were removed by gentle pipetting. Adherent cells were cultured in fresh R10 medium supplemented with 25 ng ml⁻¹ rhGM-CSF and 10 ng ml⁻¹ rhIL-4. After 7 days of culture, immature DCs were obtained by gentle pipetting. The purity of the obtained DC was greater than 98% as estimated by flow cytometry and staining of DC with anti-CD11c Ab (BD Biosciences, San Jose, CA, U.S.A.).

Various molecular and cellular assays were performed at MDS Panlabs (Bothell, WA, USA) according to their standard operation procedures (for details, see: <http://www.mdsp.com/>). Human PBMC, DC or mouse peritoneal macrophage (2×10^5 cells well⁻¹) cells were stimulated for 16 h with 25 ng ml⁻¹ LPS (from *Escherichia coli* 0127:B8, Sigma) in the presence of the test compound or the respective volume of solvent (DMSO). Supernatants were frozen at -20°C until further use and TNF- α concentrations were quantified using commercially available ELISA kits (Genzyme Techné, Cambridge, MA, U.S.A.).

Administration of drugs

Animals were kept under standard conditions in a 12 h day/night rhythm with free access to food and water *ad libitum*. All animals received humane care and the studies have been approved by the internal ethic committee in accordance with the guidelines recommended by Japanese Association of Laboratory Animal Science (JALAS).

Plasma level determinations of COMPOUND A

Male Wistar rats or female BALB/c mice (7–9 weeks old, SPF, Charles River Inc., Yokohama, Japan) were fasted overnight with free access to water prior to experiments. COMPOUND A was administered intravenously and orally to rats and mice, and the systemic blood samples were drawn at the time points specified. All blood samples were centrifuged at 600 $\times$ g for 10 min to obtain plasma samples. The partition ratio (R_b) of COMPOUND A between whole blood and plasma was measured with heparinized whole blood at 37°C for 5 min, and a small volume of COMPOUND A solution was added to give a final concentration of 1 μ g ml⁻¹. After incubation at 37°C for 15 min, the plasma concentrations of COMPOUND A were determined. COMPOUND A was extracted from the plasma with 7 ml of ether under neutral conditions, and

the organic layer was evaporated under a nitrogen stream. The residue was dissolved in a mobile phase of HPLC analysis. The sample was analyzed by an HPLC system (LC-10A; Shimadzu, Kyoto, Japan) equipped with a UV detector (SPD-10A, Shimadzu) adjusted to 356 nm. The column was a Symmetry C₁₈ (3.5 mm, 4.6 mm $\times$ 100 mm, Waters, Milford, MA, U.S.A.), and the mobile phase was composed of acetonitrile (35%) and 1% triethylamine at pH 7.4 (65%). The flow rate and column temperature were 1.0 ml min⁻¹ and 40°C, respectively.

Pharmacokinetic parameters were determined using standard noncompartmental methods. AUC was calculated by the linear trapezoidal rule. Plasma clearance (CL) was calculated as the i.v. bolus dose divided by the AUC. Blood clearance was calculated as CL R_b^{-1} . The half-life was determined by linear regression of the terminal log-linear phase of concentration–time curve. Bioavailability (BA) was estimated from the dose-adjusted ratio of AUCs after i.v. and p.o. administration.

LPS-induced TNF- α production in mice and rats

LPS-induced cytokine production in mice was performed as described (Murata *et al.*, 2003). Rats (Wistar, ♀, 7 weeks old, SPF, Charles River Inc.) were injected i.p. with 1 mg of LPS dissolved in PBS (1 ml head⁻¹). COMPOUND A, theophylline or vehicle were administered p.o. (2 ml head⁻¹) 60 min prior to LPS injection. At 2 h after LPS injection, blood was collected from the abdominal vein under slight anesthetization by i.p. injection of urethane (2 g kg⁻¹). After blood collection, rats were killed by complete bleeding. Concentration of TNF- α in plasma was measured using a commercially available ELISA kit (rat TNF alpha ELISA, Endogen).

Cockroach allergen-induced airway inflammation and hyperreactivity in mice

Sensitization and induction of the airway response In order to induce a Th2-type response, the following procedure was established in normal BALB/c mice as previously described (Campbell *et al.*, 1998; 1999). The mice were immunized with 10 μ g of cockroach allergen (Holister-Stier) in incomplete Freund's adjuvant on day 0. On day 14 the mice were given an intranasal challenge of 10 μ g of cockroach allergen in 10 μ l of diluent to localize the response to the airway. This initial intranasal challenge with antigen induced little cellular infiltrate into the lungs of the mice upon histological examination. Mice were then rechallenged 6 days later by intratracheal administration of 10 μ g of cockroach allergen in 50 μ l of sterile PBS or with PBS alone. The magnitude of leukocyte recruitment in both the vehicle control and cockroach allergen challenged mice were examined histologically. COMPOUND A was administered systemically to the mice at the time of intratracheal allergen challenge using three different doses. The mice were analyzed for the various parameters listed below at various time points post-allergen challenge.

Airway hyperreactivity (AHR) was measured using a Buxco mouse plethysmograph which is specifically designed for the low tidal volumes (Buxco, Troy, NY, U.S.A.) as previously described (Campbell *et al.*, 1998; 1999). Briefly, the mouse to be tested was anesthetized with sodium pentobarbital and intubated *via* cannulation of the trachea with an 18-gauge

metal tube. The mouse was subsequently ventilated with a Harvard pump ventilator (tidal volume = 0.4 ml, frequency = 120 breaths min⁻¹, positive end-expiratory pressure 2.5–3.0 cm H₂O) and the tail vein was cannulated with a 27 g needle for injection of the methacholine challenge. The plethysmograph was sealed and readings monitored by computer. Resistance was calculated by the Buxco software by dividing the change in pressure (Ptp) by the change in flow (F) (P_{tp}/F ; units = cm H₂O⁻¹ ml⁻¹ s⁻¹) at two time points from the volume curve based upon a percentage of the inspiratory volume. After determining a dose–response curve (0.001–0.5 mg), an optimal dose was chosen, 0.1 mg of methacholine. This dose was used throughout the rest of the experiments in this study. After the methacholine challenge, the response was monitored and the peak airway resistance recorded as a measure of AHR.

Morphometric analysis of peribronchial and airway eosinophil accumulation was performed. Lungs from mice immunized and challenged with cockroach allergen or vehicle were preserved with 1 ml of 4% paraformaldehyde at various time points post-challenge. The fixed lungs were embedded in paraffin and multiple 50 μ m sections were differentially stained with Wright-Giemsa for the identification of eosinophils and viewed at $\times$ 1000. The individual eosinophils were counted from 100 high-powered fields (HPF) per lung at each time point using multiple step sections of lung. Only the eosinophils in the peribronchial region were counted, this assured the enumeration of only those eosinophils within or immediately adjacent to an airway. The inflammation observed in this model was completely associated with the airway with little or no alveolitis.

Cytokines were quantitated from homogenized (PBS with 0.05% Triton X-100 nonionic detergent) lung aqueous extracts using a double ligand ELISA system. The murine ELISAs were set up using standardized antibodies purchased from R&D systems. Detected protein at concentrations above 10 pg ml⁻¹ were specific and did not crossreact to any other cytokine.

Ovalbumin (OVA)-induced airway inflammation in rats

Sensitization to OVA (albumin, chicken egg, Grade V, #A-5503, Sigma) was performed by i.p. injection of OVA/alum (OVA (1 mg rat⁻¹) in Al(OH)₃ (100 mg rat⁻¹) in saline) on days 0 and 14 (once a day, i.p.). Rats in the sham group were sensitized by i.p. injection of alum (Al(OH)₃ (100 mg rat⁻¹) in saline). On days 20 and 21, rats were challenged by OVA inhalation for 30 min. Sham-treated rats were exposed to saline aerosol mist for 30 min. On day 22, BAL was carried out. After terminal anesthesia, the trachea was exposed and a tube connected to two syringes (50 and 15 ml) *via* a three-way cock was inserted. The airways were washed three times with 5 ml 0.1% BSA (albumin, bovine, fraction V, #A-2153, Sigma) in saline, pumped into the lungs by a 50 ml syringe. BAL fluid (BALF) was collected by a 15 ml syringe. The amount of BALF recovered was recorded to calculate the number of cells per BALF. Collected BALF was transferred to a 15 ml tube, and spun down at 4°C. The supernatant was removed and cells were resuspended to 1 ml of BSA in saline (0.1%). The cell suspension was diluted to one-tenth in Turk's stain solution (Nacalai Tesque). The number of total cells in the sample was counted under the microscope using a hemocytometer. Cytospin specimens were stained with May-Gruenwald's

(Merck, Darmstadt, Germany) and Giemsa's solution (Merck) for leukocyte typing. The distribution of each cell population (neutrophils, eosinophils, macrophages, lymphocytes and others) was counted under microscopy by counting more than 200 cells.

Induction of ear edema in mice

Phorbol 12-myristate 13-acetate (PMA, 5 μ g Sigma) or 500 μ g arachidonic acid (AA, Sigma) was dissolved in 20 μ l of acetone and applied to the inner and outer surfaces of the right ear of each mouse (BALB/c, δ , 7–8 weeks old, SPF, Charles River Inc.). In the model of delayed-type hypersensitivity (DTH)-induced edema, mice were sensitized by applying 30 μ l of 0.5% dinitrofluorobenzene (DNFB) dissolved in acetone:olive oil (volume:volume = 4:1) to their shaved abdomen on days –7 and –6. On day 0, mice were challenged by topical application of 20 μ l of 0.3% DNFB in acetone:olive oil (4:1) to the right ear. In all models, acetone or acetone:olive oil was applied to the left ear as a control. Ear thickness was measured at 0, 3, 6, 9, and 24 h after PMA application, at 0, 1, 3, and 6 h after AA application, or at 0, 24, and 48 h after DNFB challenge using a calibrated thickness gauge (Mitsutoyo, Tokyo, Japan) under anesthetization with ether. Ear edema was expressed as $(R-L)-(R_0-L_0)$, where R_0 and L_0 represent the thickness of the right and left ears, respectively, at the beginning of the experiment (0 h), and R and L stand for the thickness values obtained at the respective time points.

Carrageenan-induced pleurisy in mice

Mice (Balb/c, φ , 7–8 weeks old, SPF, Charles River Inc.) received a single intrapleural injection with 0.2 ml of sterile saline containing κ -carrageenan (0.25%) (Wako Chemicals) under anesthetization with ether. COMPOUND A, dexamethasone, or vehicle were administered p.o. (0.2 ml head⁻¹) 60 min prior to carrageenan injection. At 4 h after injection, mice were killed and pleural fluid was collected by washing the pleural cavity twice with 1 ml PBS. Cell stains were performed as described above.

Drugs

COMPOUND A (7-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-5-[(3*S*)-3-piperidinyl]-1,4-dihydro-2*H*-pyrido[2,3-*d*][1,3]oxazin-2-one hydrochloride) and COMPOUND B (the enantiomeric mixture of COMPOUND A) were synthesized in the laboratories of Bayer Yakuhin, Ltd (Kyoto, Japan). Theophylline (Nacalai Tesque, Kyoto, Japan), dexamethasone (dexamethasone 21-phosphate disodium salt, Sigma, MO, U.S.A.) and cyclosporin A (CsA, Sandimmune, Novartis, CH, U.S.A.) were obtained from the supplier indicated. For administration to animals, compounds were suspended in 10% cremophor (Nacalai Tesque) in saline, which was used as the vehicle in all experiments.

Statistical analysis

If not otherwise stated, data are expressed as mean values $\pm$ s.e.m. Statistical differences of data sets were analyzed using one-way ANOVA and differences between groups were assessed by Dunnett's method or, if applicable, by Student's

t-test using commercially available statistics software (Graph-Pad Software, Inc., San Diego, U.S.A.). *P*-values < 0.05 were considered statistically significant. Details are given in each figure legend.

Results

Inhibition of IKK- β

During a chemical optimization program of small-molecule IKK- β inhibitors derived from HTS (Murata *et al.*, 2003; 2004a, b), COMPOUND A was identified as a potent derivative (Figure 1a). COMPOUND A inhibited kinase activity of human recombinant IKK- β with a K_i of 2 nM for ATP (Figure 1b) and 4 nM for the substrate GST-I κ B α (1–54) (Figure 1c). Double reciprocal (data not shown) and Dixon plots indicate that the mode of inhibition by COMPOUND A is competitive to ATP (Figure 1b), but noncompetitive for the substrate, GST-I κ B α (1–54) (Figure 1c). COMPOUND A inhibited IKK- α at higher concentrations (K_i for ATP: 135 nM), but did not inhibit (IC_{50} > 10 μ M) the protein kinases IKK3, MKK4, MKK7, ERK-1, Syk, Lck, Fyn, PI3K γ , PKA (nonselective), and PKC (nonselective). Furthermore, COMPOUND A did not inhibit (IC_{50} > 10 μ M) the phospholipases PLA2-I and PLC, the proteases caspase 1, 3, 4, 6, 7, 8, MMP-1, 2, 3, 7, 9, the phosphatases PP2B, PTP1C, CD45, PTP1B, PTP from T-cells or neutral sphingomyelinase. These results indicate that COMPOUND A is a potent and selective IKK- β inhibitor.

To test whether COMPOUND A also inhibits the physiologically stimulated IKK-complex, the complex was precipitated from cell lysates of TNF- α -stimulated A549 cells using anti-IKK- α antibodies (Figure 1d). COMPOUND A inhibited the kinase activity of the IKK-complex precipitated, in a concentration-dependent manner with similar potency (Figure 1e). This demonstrates that COMPOUND A not only inhibits the homodimer of human recombinant IKK- β in a cell-free system, but also the physiologically activated IKK complex.

COMPOUND A inhibits IKK- β -dependent signal transduction

Next we tested whether the selectivity of COMPOUND A we observed at isolated targets would translate to specific inhibition of the NF- κ B signaling pathway in living cells. Therefore, we exposed A549 cells to TNF- α to activate the IKK complex and monitored I κ B α phosphorylation and ensuing degradation in the presence or absence of the IKK- β inhibitor. TNF- α stimulation induced a rapid induction of I κ B α phosphorylation which peaked at 5 min and typically disappeared quickly due to I κ B α degradation (Figure 2a, b). At 30 min after stimulation, newly synthesized I κ B α appeared with a significant phosphorylation signal being evident at 60 min (Figure 2a, b). COMPOUND A inhibited I κ B α phosphorylation and degradation in cells stimulated by TNF- α in a concentration-dependent manner (Figure 2a, b).

In line with these results, COMPOUND A inhibited TNF- α -induced production of the chemokine RANTES after 24 h, with an IC_{50} of 40 nM in A549 cells (Figure 2c). On the transcriptional level, COMPOUND A also inhibited TNF- α -

induced NF- κ B-dependent reporter gene activation in HEK293 cells, with an IC_{50} of 240 nM (Table 1). The signal pathway selectivity of COMPOUND A was tested in other reporter gene assays. COMPOUND A did not inhibit PMA-induced AP-1 activation in MRC-5 cells ($IC_{50} > 10 \mu M$) as shown in Table 1. COMPOUND A inhibited PMA/calcium ionophore-induced NF-AT-dependent reporter gene transcription in Jurkat cells only at higher concentrations (IC_{50} : 4 μM), but inhibited PMA/calcium ionophore-induced NF- κ B-depen-

dent reporter gene induction in the same cells with an IC_{50} of 148 nM (Table 1). These results indicate that COMPOUND A selectively interferes with the NF- κ B signaling cascade in living cells.

COMPOUND A inhibits IKK- β -dependent NF- κ B activation in various cells

A large number of extracellular stimuli including TNF- α , LPS, TCR plus costimulation, or PMA activate the IKK complex. To further explore its anti-inflammatory potential, the effects of COMPOUND A in various cell assays representing inflammatory reactions were investigated. COMPOUND A inhibited TNF- α -induced VCAM-1 expression on HUVECs with an IC_{50} of 85 nM. Moreover, COMPOUND A inhibited LPS-induced TNF- α release in human PBMCs (IC_{50} : 47 nM, Figure 2d), DCs (IC_{50} : 220 nM, Table 1), and mouse peritoneal macrophages (IC_{50} : 80 nM, Table 1). As shown in Table 1, COMPOUND A inhibited in human PBMCs also LPS-induced IL-1 β (IC_{50} : 52 nM) and IL-6 release (IC_{50} : 18 nM). Furthermore, ConA-induced release of IL-2 (IC_{50} : 0.27 μM), IFN- γ (IC_{50} : 0.3 μM), IL-4 (IC_{50} : 0.14 μM), IL-5 (IC_{50} : 0.5 μM), and IL-10 (IC_{50} : 0.3 μM) as shown in Table 1. COMPOUND A inhibited LPS-induced mouse B-cell and ConA-induced T-cell proliferation with IC_{50} 's of 65 nM and 0.62 μM , respectively (Table 1).

Collectively, COMPOUND A inhibited NF- κ B activation in response to various stimuli and with regard to a variety of readout parameters, all representing immune cell activation.

Pharmacokinetic profile of COMPOUND A in rodents

To test the anti-inflammatory activity of COMPOUND A in animal models of asthma, we investigated the pharmacokinetic properties of COMPOUND A in mice and rats. The dose-normalized plasma concentrations obtained for COMPOUND A after intravenous and oral administrations are summarized in Figure 3a and c for mice and rats, respectively. After intravenous administration of COMPOUND A at a dose of 2 mg kg⁻¹, the plasma concentrations declined with half-lives of 1.3 and 2.1 h in mice and rats, respectively. Both

Figure 1 Structure and *in vitro* activity of COMPOUND A. (a) Structure (7-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-5-[(3S)-3-piperidinyl]-1,4-dihydro-2H-pyrido[2,3-d][1,3]oxazin-2-one hydrochloride). (b, c) Kinase activity of human recombinant IKK- β (7 nM) was measured at various concentrations of [γ -³²P]ATP and GST-I κ B α (1-54) substrates in the presence of COMPOUND A. Dixon plots were obtained from the initial velocity of IKK- β -catalyzed phosphorylation. (d) In all, 100 μ g of whole cell lysate from A549 cells was subjected to immunoprecipitation by using 0.2, 1, or 2 μ g of anti-IKK- α antibody. Cell lysate (Lys), flow through fraction of IP (FT) and immunoprecipitated sample (IP) were subjected to SDS-PAGE and immunoblot analysis by using anti-IKK- α or anti-IKK- β antibodies. To the three lanes on the right side, recombinant proteins expressed in the baculovirus system were applied. α : His-tagged IKK- α , β : His-tagged IKK- β , NIK: FLAG-tagged NIK. (e) A549 cells were stimulated with TNF- α (100 ng ml⁻¹) for 15 min. NS indicates nonstimulated control. After lysis, the IKK complex was quantitatively precipitated from the soluble fraction using 2 μ g of anti-IKK- α antibody and the kinase reaction was performed with GST-I κ B α (1-54) and [γ -³²P]ATP (5 μ M) as substrates in the presence of vehicle (performed in duplicate) or the indicated concentrations of COMPOUND A.

species investigated showed relatively large distribution volumes of 5.30 and 6.90 l kg⁻¹. CL was estimated to be 2.44 and 2.31 l h⁻¹ kg⁻¹ for mice and rats, respectively. Considering

Table 1 Summary of cellular activities of COMPOUND A

Stimulus	Cells	Readout	IC ₅₀ (nM)
TNF- α	HEK293	NF- κ B transactivation	240
PMA	MRC5 cells	AP-1 transactivation	> 10,000
PMA/Ca	Jurkat cells	NF-AT transactivation	4090
PMA/Ca	Jurkat cells	NF- κ B transactivation	148
TNF- α	HUVECS	VCAM-1	85
LPS	Human DCs	TNF- α	220
LPS	Mouse macrophages	TNF- α	80
LPS	Human PBMCs	IL-1 β	52
LPS	Human PBMCs	IL-6	18
Concavalin A	Human PBMCs	IL-2	272
Concavalin A	Human PBMCs	IFN- γ	306
Concavalin A	Human PBMCs	IL-4	140
Concavalin A	Human PBMCs	IL-5	502
Concavalin A	Human PBMCs	IL-10	308
LPS	Mouse B cells	Proliferation	65
Concavalin A	Mouse T cells	Proliferation	617

the hepatic blood flow rate in each species, COMPOUND A can be categorized as a moderate clearance compound. Following oral administration at 10 mg kg⁻¹ to mice and rats, the normalized C_{max} was found to be 0.0301 mg l⁻¹ (76 nM) and 0.0306 mg l⁻¹ (77 nM), respectively. The normalized AUC was 0.0998 mg h l⁻¹ in mice and 0.223 mg h l⁻¹ in rats. Bioavailability was 36 and 69%, respectively. In summary, these results indicate that both in mice and rats COMPOUND A is orally available and shows a desirable pharmacokinetic profile.

Inhibition of pro-inflammatory mediator release in rodents by COMPOUND A

Based on the favorable pharmacokinetic profile of COMPOUND A and its high efficiency in abrogating NF- κ B activation *in vitro*, we next investigated the oral efficacy of the IKK- β inhibitor in rodent models of systemic inflammatory response syndrome (SIRS), a condition which is characterized by massive inflammatory mediator release and ensuing multi-organ failure, eventually leading to death. The SIRS reaction was triggered by challenging animals with LPS, an inflammatory

Figure 2 Cellular activity of COMPOUND A. A549 cells were treated for 1 h with vehicle or COMPOUND A at the indicated concentration. Thereafter, cells were stimulated with TNF- α (10 ng ml⁻¹) for the time indicated and lysed. Proteins were separated by SDS-PAGE, transferred to a membrane and probed with antibodies specific for phosphorylated I κ B α (a) or I κ B α (b). (c) Effects of COMPOUND A on TNF- α -induced RANTES production in A549 cells. A549 cells were pre-incubated with COMPOUND A for 1 h and then stimulated with 100 ng ml⁻¹ TNF- α for 24 h. The culture supernatant was collected and concentration of RANTES was determined by FIA. (d) Effects of COMPOUND A on LPS-induced TNF- α release in human PBMCs. PBMCs freshly prepared from healthy donors were preincubated with COMPOUND A for 1 h and then stimulated with 3 ng ml⁻¹ LPS for 7 h. The culture supernatant was collected and concentration of TNF- α was determined by ELISA.

stimulus leading to strong NF- κ B activation. In mice a single dose of COMPOUND A given p.o. 60 min before LPS challenge inhibited TNF- α production in a dose-dependent manner with ED₅₀ = 9.1 mg kg⁻¹ (Figure 3b). In rats, COMPOUND A given p.o. 60 min before LPS challenge inhibited TNF- α production in a dose-dependent manner with ED₅₀ = 6.6 mg kg⁻¹ (Figure 3d). Theophylline showed marked inhibition at 30 mg kg⁻¹ in both species (Figure 3b,d). These results indicate that COMPOUND A showed comparable efficacy to a nonselective PDE inhibitor.

Cockroach allergen-induced airway inflammation and hyperreactivity model in mice

Allergen-induced airway inflammation and hyperreactivity have a significant impact on asthmatic symptoms. We therefore evaluated COMPOUND A in a Th2-driven allergy model triggered by cockroach allergen immunization and ensuing intranasal and intratracheal allergen challenge (Campbell *et al.*, 1998). The enantiomeric mixture of COMPOUND A, termed COMPOUND B, inhibited AHR at 24 h post-challenge at doses equal to or higher than 10 mg kg⁻¹ (corresponding to 5 mg kg⁻¹ of the active ingredient, COMPOUND A, Figure 4a) when given p.o. at the time of intratracheal allergen challenge. The intensity of AHR is often associated with the accumulation of leukocytes, especially eosinophils, around the airways of the allergic mice. The effect of COMPOUND B on eosinophil accumulation was tested at an intermediate dose of 25 mg kg⁻¹, which significantly inhibited AHR (Figure 4a). Histological examination of the lungs of allergic animals clearly demonstrated that COMPOUND B was capable of significantly inhibiting the overall inflammatory response and significantly reducing eosinophil accumulation (Figure 4b). In fact, the number of recruited eosinophils present after administration of COMPOUND B demonstrated that the peribronchial eosinophil response was nearly abrogated at 24 h post-challenge (Figure 4c). To determine whether there was a reduction in specific cytokine mediators that have previously been identified to be increased in allergic asthmatic responses, we examined pulmonary cytokine levels. Lungs from allergic mice were homogenized and the debris free supernatant was subjected to ELISA analyses of macrophage-associated (IL-12, TNF- α) and Th2 cell-associated (IL-4, IL-5) cytokines. The data shown in Figure 4d illustrate that COMPOUND B at 25 mg kg⁻¹, significantly reduced IL-4, IL-5, and IL-12. Thus, the cytokine responses appeared to follow the hyperreactivity results examined in Figure 4a.

Figure 3 Pharmacokinetic properties of COMPOUND A and efficacy in mechanistic animal model. (a, c) Dose-normalized plasma concentration profiles of COMPOUND A after intravenous (2 mg kg⁻¹) and oral (10 mg kg⁻¹) administrations in mice (a) or rats (c). (b, d) Increase in plasma level of TNF- α was induced by intraperitoneal injection of LPS (200 μ g mouse⁻¹ (b) or 1 mg rat⁻¹ (d), i.p.). COMPOUND A, theophylline, or vehicle were given p.o. 60 min before the LPS injection. Each column indicates the mean and s.e.m. of 7–8 animals. Statistical differences between vehicle control and COMPOUND A groups were analyzed using one-way ANOVA and Dunnett's method (** P < 0.01). For theophylline, data were analyzed by Student's *t*-test (### P < 0.01). V: vehicle, N: no stimulation with LPS.

Protection from OVA-induced lung inflammation by COMPOUND A

To fully elaborate the IKK- β inhibitor's potential in a disease-relevant model of chronic inflammation, we investigated the influence of COMPOUND A on leukocyte infiltration in OVA-sensitized and -challenged rats, a widely used standard

model for antiasthma drug evaluation. Male BN rats were sensitized with i.p. injection of OVA and alum on days 0 and 14, and then inhaled 1% OVA in saline on days 20 and 21. BAL was performed on day 22. COMPOUND A was given p.o. from days 0 to 21 (b.i.d.). Dexamethasone was given p.o. from days 0 to 7 and from days 14 to 21 (b.i.d.). COMPOUND A reduced the number of migrated eosinophils by 75% at a dose of 0.3 mg kg⁻¹ time⁻¹ and higher (Figure 5a). COMPOUND A also inhibited the migration of neutrophils, even at 0.3 mg kg⁻¹ time⁻¹ (Figure 5a). Dexamethasone (0.3 mg kg⁻¹ time⁻¹) inhibited the number of migrated eosinophils and neutrophils markedly. COMPOUND A did not decrease the body weight even at the 30 mg kg⁻¹ time⁻¹, although dexamethasone inhibited severely the body weight at 0.3 mg kg⁻¹ time⁻¹ (Figure 5b). These results indicate that COMPOUND A shows an efficacy similar to dexamethasone in a rat model of asthma.

COMPOUND A is efficacious in models of edema formation

We next set out to examine whether COMPOUND A would be active in other models of inflammation and studied the effect of COMPOUND A pretreatment in three different models of edema.

PMA-induced ear edema depends on LTB₄-induced neutrophil migration and activation. LTB₄ production itself might not be NF- κ B-dependent, but neutrophil migration completely depends on NF- κ B activation, such as induction of ICAM-1 expression on endothelial cells by TNF- α production. The ear edema increased by 25 h after PMA (10 μ g ear⁻¹) application. Ear thickness was measured at 0, 3, 6, 9, and 24 h after PMA application. COMPOUND A, dexamethasone, and vehicle were given p.o. 60 min before PMA challenge. As shown in Figure 6a, COMPOUND A inhibited PMA-induced ear edema in a dose-dependent manner with an ED₅₀ of about 0.3 mg kg⁻¹. The efficacy was comparable to that of dexamethasone.

AA-induced ear edema depends on COX-2 expression, which is regulated by NF- κ B activation. PGE₂ produced by COX-2 induction causes vascular permeability. AA-induced edema reflects permeability without cell migration. Ear edema reached a plateau 1 h after AA (500 μ g ear⁻¹) application. Ear thickness was measured at 0, 1, 3, and 6 h after AA challenge. COMPOUND A, dexamethasone, and vehicle were given p.o. 60 min before the AA application. As shown in Figure 6b,

Figure 4 Effect of COMPOUND B (CPD B) on cockroach allergen (CRA)-induced airway inflammation and hyperreactivity in mice. COMPOUND B was given orally at the time of allergen challenge. (a) AHR was measured at 24 h post-challenge using a Buxco mouse plethysmograph. After a methacholine challenge via the cannulated tail vein, the response was monitored and the relative peak airway resistance was recorded as a measure of AHR. (b, c) At 24 h post challenge, lungs were preserved with 1 ml of 4% paraformaldehyde. The fixed lungs were embedded in paraffin and multiple 50 μ m sections were differentially stained with Wright-Giemsa for the identification of eosinophils (b). The individual eosinophils were counted from 100 HPFs per lung using multiple step sections of lung. The number of eosinophils at a dose of 25 mg kg⁻¹ in comparison to vehicle is shown (c). (d) Cytokine levels in lungs from allergic mice 24 h post challenge. Data were analyzed using ANOVA (* P < 0.05).

Figure 5 Effect of COMPOUND A on OVA-induced airway inflammation in rats. Male rats were sensitized by i.p. injection of OVA and alum on days 0 and 14, then were inhaled with 1% OVA in saline on days 20 and 21. BAL fluid was collected on day 22 and the number of different cells was determined. COMPOUND A was given p.o. from days 0 to 21 (b.i.d.). Dexamethasone was given p.o. from days 0 to 7 and from days 14 to 21 (b.i.d.). Data show the mean and s.e.m. of eight rats. (a) Number of cells in BAL fluid. Statistical differences between vehicle control- and COMPOUND A-administered groups were analyzed by one-way ANOVA and Dunnett's multiple comparison test (* $P < 0.05$, ** $P < 0.01$). Statistical differences between vehicle control- and dexamethasone-administered or sham group data were analyzed by Student's *t*-test (# $P < 0.05$, ## $P < 0.01$). (b) Shows body weight development. V: vehicle, Dex: dexamethasone, Sham, no sensitization with OVA.

COMPOUND A inhibited AA-induced ear edema in a dose-dependent manner with a MED of 0.3 mg kg^{-1} . The overall efficacy of COMPOUND A was slightly weaker than that of dexamethasone.

Delayed type hypersensitivity (DTH) was induced by repeated application of DNFB. DTH represents type IV allergy, but it has also been reported that DTH contributes to the pathogenesis of severe asthma. DTH-induced ear edema depends on migrated cytotoxic T cells. The migration of

cytotoxic T cells is regulated in a complicated manner; however, NF- κ B activation absolutely contributed to T-cell extravasation by controlling the expression of adhesion molecules on endothelial cells upon inflammatory activation. Ear edema in this model became evident at 24–48 h after DNFB (0.3%) challenge of DNFB-sensitized mice. COMPOUND A, cyclosporin A, or vehicle were given p.o. 60 min before the challenge. As shown in Figure 6c, COMPOUND A inhibited DNFB-induced ear edema in a dose-dependent manner with an ED₅₀ of about 0.3 mg kg^{-1} at all time points investigated. This clearly shows that the inhibition of IKK- β is an efficient strategy to block edema formation under various inflammatory conditions.

Effect of COMPOUND A on leucocyte trafficking

Finally, we investigated the influence of IKK- β inhibition on leukocyte trafficking. For that purpose, neutrophilia was induced by carrageenan injection into the pleural cavity of mice and infiltrating leukocytes were counted. Figure 7 clearly shows that COMPOUND A led to a significant reduction of both total cells (Figure 7a) and neutrophils (Figure 7b) at both 0.3 and 1 mg kg^{-1} . Maximal reduction of neutrophilia achieved by COMPOUND A treatment was about 50% and similar to that observed in mice pretreated with dexamethasone.

Discussion

The data presented show that COMPOUND A is a highly selective and competitive inhibitor for the IKK- β subunit of the IKK complex. While an inhibition of another of the about 500 protein kinases in the human body cannot be excluded, the high level of selectivity *versus* IKK- α (> 50 -fold), numerous other kinases (> 100 -fold), as well as a broad spectrum of enzymes and receptors representing various pharmacological target classes as well as the high potency suggest that COMPOUND A does indeed interact specifically only with IKK- β .

From the standpoint of the toxicity profile of a potential therapeutic agent, selectivity for IKK- β over IKK- α should be advantageous, because IKK- β has been shown to play a critical role in I κ B α phosphorylation and NF- κ B activation in response to proinflammatory stimuli. In contrast, IKK- α is thought to play a minor role in inflammation, but rather in skin and B-cell lymphoid organ development (Hu *et al.*, 1999; 2001; Takeda *et al.*, 1999; Senftleben *et al.*, 2001; Muller & Siebenlist, 2003; Sil *et al.*, 2004). Another concern in targeting IKK- β is that inhibition of IKK- β might result in massive apoptosis as seen in the embryonic phenotype of IKK- β k.o. mice or in enterocytes deleted for IKK- β after an inflammatory stimulus (Li QT *et al.*, 1999; Li ZW *et al.*, 1999; Tanaka *et al.*, 1999; Chen *et al.*, 2003). Furthermore, IKK- β -deficient B-cells show defects in survival and proliferation (Pasparakis *et al.*, 2002; Li *et al.*, 2003). Importantly, no signs of liver damage, the most obvious safety concern stemming from the phenotype of the k.o. mice, have been observed in mice and rats under COMPOUND A treatment for several weeks (data not shown). Similar observations were made with another IKK- β inhibitor which showed no obvious signs of toxicity given over several weeks (McIntyre *et al.*, 2003). Perhaps pharmacological inhibition of IKK- β may not be as complete

as deletion of the gene and allow a sufficient level of basal NF- κ B activity to prevent apoptosis, while still being sufficient to limit the inflammatory activity of NF- κ B.

Selectivity of COMPOUND A was shown for the NF- κ B pathway by inhibition of phosphorylation of I κ B α , whereas other signal transduction cascades such as the AP-1 remained unaffected. This is especially important, because there is a hierarchically structured cascade of kinases working sequentially in many of these signaling pathways such that blocking any of the upstream kinases would have resulted in the inhibition of a given measured end point. For instance, inhibition of c-Jun NH₂-terminal kinase, MKK4/7, or mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, etc. would have resulted in the inhibition of c-Jun phosphorylation. Lack of effects on these signal transduction pathways in cells, therefore, provides additional evidence for the selectivity of COMPOUND A for the catalytic subunits of IKK.

In line with genetic evidence (Li QT *et al.*, 1999; Li ZW *et al.*, 1999; Tanaka *et al.*, 1999), COMPOUND A inhibited the productions of various cytokines such as TNF- α , IL-1 β , IL-2, IL-4, IL-5, and IL-10 in response to LPS and mitogenic stimuli in primary cells as well as cell lines and inhibited the proliferation of B and T cells. Given the oral bioavailability, it was no surprise that COMPOUND A was also active in the rodent models of LPS-induced TNF- α release in mice and rats, with ED₅₀'s in the range of 5–10 mg kg⁻¹. This suggested that, based on the pivotal role of IKK- β in inflammatory response signaling, a specific IKK- β inhibitor could have a broad therapeutic applicability in numerous inflammatory diseases such as asthma. Indeed, COMPOUND A showed efficacy in two animal models of chronic pulmonary inflammation, the cockroach allergen-induced airway inflammation and hyperreactivity model in mice and the OVA-induced lung inflammation model in rat.

In the cockroach allergen-induced airway inflammation and hyperreactivity model, one of the most striking observations was the reduction of eosinophil accumulation within the lung, which was paralleled by the pulmonary levels of IL-5 during the response (Figure 4). Clearly, IL-5 has been established as an important factor for eosinophil maturation and release into peripheral circulation (Gleich, 2000). COMPOUND A significantly reduced eosinophil accumulation in the treated animals and the cytokine levels of IL-4 and IL-5 compared to

Figure 6 Effect of COMPOUND A on induction of ear edema in mice. (a) Ear edema was induced by a topical application of PMA. COMPOUND A, dexamethasone or vehicle were given p.o. 60 min before the PMA application. Ear thickness was measured at 0, 3, 6, 9, and 24 h after the PMA application. (b) Ear edema was induced by topical application of AA. COMPOUND A, dexamethasone or vehicle were given p.o. 60 min before AA (500 μ g ear⁻¹) application. Ear thickness was measured at 0, 1, 3, and 6 h after the AA application. (c) Mice were sensitized with DNFB. Mice were then challenged with topical application of DNFB on day 0. Ear thickness was measured at 0, 24, and 48 h after the DNFB challenge. COMPOUND A, cyclosporin A or vehicle were given p.o. 60 min before the DNFB challenge. Each column indicates the mean and s.e.m. of five animals. Statistical differences between vehicle control and COMPOUND A-treated groups were analyzed using one-way ANOVA and Dunnett's method (* P <0.05, ** P <0.01). For dexamethasone and cyclosporin A, data were analyzed by Student's *t*-test (## P <0.01, ### P <0.001). Dex: dexamethasone. CsA: cyclosporin A.

Figure 7 Effect of COMPOUND A on carrageenan-induced pleurisy in mice. Carrageenan was injected into the pleural cavity of mice under light anesthesia. Pleural lavage was carried out 4 h after the carrageenan injection and the number of total cells (a) and neutrophils (b) was determined. COMPOUND A, dexamethasone, or vehicle were administered p.o. 60 min before the carrageenan injection. Each column indicates the mean and s.e.m. of five animals. Statistical differences between vehicle control and COMPOUND A-treated groups were analyzed using one-way ANOVA and Dunnett's method (** $P < 0.01$). For dexamethasone data were analyzed by Student's *t*-test (### $P < 0.001$). Dex: dexamethasone, NT: no carrageenan injection, ND: not done.

vehicle control. This supports previous studies that have identified a role of the NF- κ B pathway for the generation of Th2-type cytokines in activated lymphocyte populations (Boothby *et al.*, 2001), and suggests that inhibiting the activation of the NF- κ B-mediated pathway may be a legitimate method to block the mechanisms responsible for induction of AHR.

Leukocyte recruitment to inflammatory sites of the body in response to carrageenan or OVA was also diminished by COMPOUND A (Figures 5 and 7). In line with our data, both stimuli have been shown to lead to NF- κ B activation (D'Acquisto *et al.*, 1999; Poynter *et al.*, 2002) and both models are sensitive to inactivation of the IKK/NF- κ B pathway (Yang *et al.*, 1998; Donovan *et al.*, 1999). Most likely, the abrogation

of NF- κ B activation affects leukocyte recruitment on different levels: Upregulation of surface adhesion molecules critical for leukocyte trafficking, the production of chemoattractants, and leukocyte migration in response to the activation of chemoattractants are all, at least in part, NF- κ B-dependent (Baeuerle & Baichwal, 1997).

Indeed, several lines of evidence indicate that activated NF- κ B contributes to asthma pathophysiology and that inhibition of NF- κ B activation will improve asthma symptoms. Mice deficient in the p50 subunit of NF- κ B were incapable of mounting an eosinophilic airway response as compared to wild-type mice. Also, p50-deficient mice lack the production of IL-4, IL-5, IL-13, and eotaxin, which are believed to play distinct roles in asthma pathogenesis. CD4-positive T cells from p50 $^{-/-}$ mice failed to induce Gata3 expression, a transcription factor present in Th2 but not Th1 cells, and plays a critical role in Th2 differentiation and allergic airway inflammation *in vivo* (Das *et al.*, 2001). Additionally, p50-deficient mice were unable to produce MIP-1 α and β , two chemokines important for T-cell recruitment to inflammatory sites (Yang *et al.*, 1998; Das *et al.*, 2001). In addition to these observations in gene-targeted mice, activity of NF- κ B has been shown to be increased in airway cells from asthmatic patients (Hart *et al.*, 1998), and Der p1, the major allergen from dust mite, has been shown to activate NF- κ B in bronchial epithelial cells from patients suffering from allergic asthma (Stacey *et al.*, 1997). Many proteins and factors known to be involved in airway inflammation, such as IL-1, TNF- α , GM-CSF, RANTES, MCP-3, iNOS, ICAM-1, and VCAM-1, are regulated by NF- κ B (Barnes & Adcock, 1997). In line with this, COMPOUND A effectively abrogated the release of monokines, lymphokines, and chemokines, and blocked the upregulation of adhesion molecules (c.f. Table 1).

Presumably, the most supportive evidence, however, for the huge therapeutic potential of an IKK- β inhibitor is based on the long-term experience of the clinical use of GCs, which are by far the most effective anti-inflammatory treatment for asthma. GCs inhibit airway inflammation by directly interacting with and inhibiting the activity of the transcription factors NF- κ B and AP-1 (De Bosscher *et al.*, 2003; Leung & Bloom, 2003). Thus, inhibition of NF- κ B activation should result in an anti-inflammatory effect similar to or superior to that of steroids. First experimental data support this hypothesis and demonstrate that COMPOUND A is efficacious in reducing airway inflammation in animal models of asthma. Furthermore, in contrast to dexamethasone, COMPOUND A did not result in a decreased weight development when given over a couple of weeks. Inhibition of weight increase is used as one marker to monitor the side effects of steroids (Belvisi *et al.*, 2001). In addition, based on what is known about the mechanism of glucocorticoid resistance, one would expect that IKK- β inhibitors which target NF- κ B more directly and using a different mechanism work in steroid-insensitive asthma. Currently, steroid insensitivity is thought to originate either from an increased activation/expression of AP-1 or NF- κ B proteins, which cannot be sufficiently transrepressed by GC-bound GR (De Bosscher *et al.*, 2003), or increased expression of an alternatively spliced, dominant-negative GR (GR- β). Thus, an IKK- β inhibitor would be able to provide a benefit for this difficult-to-treat patient group.

Inhibition of IKK- β obviously is also very efficient in blocking the formation of edema under various conditions.

Mechanistically, the protection from AA-induced edema formation may occur *via* blockade of AA-triggered COX2 induction. COX2 induction has been shown to be NF- κ B-dependent under various conditions (Newton *et al.*, 1997; Inoue & Tanabe, 1998), and AA has been reported to lead to NF- κ B activation (Camandola *et al.*, 1996). In addition, COX2 is known to be causally involved in AA-triggered edema formation, since COX2 inhibitors were reported to be protective in the same murine model we used here (Puignero & Queralt, 1997). Similarly, PMA-induced edema formation also has a COX2 component (Puignero & Queralt, 1997) and therefore it is not surprising that pretreatment of mice with COMPOUND A also resulted in abrogation of edema formation in response to PMA application (c.f. Figure 6a). Interestingly, edema induced by DTH which depends on T cells was also blocked by COMPOUND A, suggesting a critical role for NF- κ B in the development of DTH. Indeed, indirect evidence in a similar mouse model of DTH *in vivo* (Elliott *et al.*, 1999) exists, which is in accordance with our observation.

COMPOUND A is not the only IKK- β inhibitor known to date (Karin *et al.*, 2004). Recently, various compounds called PS-1145 (Hideshima *et al.*, 2002; Castro *et al.*, 2003), BMS-345541 (Burke *et al.*, 2003; MacMaster *et al.*, 2003; McIntyre *et al.*, 2003; Townsend *et al.*, 2004), SC-514 (Kishore *et al.*, 2003; Baxter *et al.*, 2004), and others (Heckmann *et al.*, 2004;

Tegeder *et al.*, 2004) have been described as IKK- β inhibitors. Although neither being as potent nor being as selective for the β -isoform of IKK as COMPOUND A, the results obtained with these tool compounds representing several additional chemical classes of drug-like molecules impressively underline the potential IKK- β inhibitors might have for diseases such as multiple myeloma (Hideshima *et al.*, 2002), rheumatoid arthritis (McIntyre *et al.*, 2003), graft rejection (Townsend *et al.*, 2004), colitis (MacMaster *et al.*, 2003), HIV1-associated disorder (Heckmann *et al.*, 2004), or inflammatory and neuropathic pain (Tegeder *et al.*, 2004).

Collectively, COMPOUND A is a potent and selective IKK- β inhibitor fulfilling the expectation of being anti-inflammatory *in vitro* and *in vivo*, and might pave the way for the development of IKK- β inhibitors as a novel class of antiasthma and anti-inflammatory drugs. Taken together, these findings underline the crucial role NF- κ B plays in the inflammatory cascade leading to pulmonary inflammation in asthma and make the upstream NF- κ B regulator, IKK- β , a promising target for the development of novel anti-inflammatory therapeutics of chronic inflammatory lung diseases.

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