Treatment of Allergic Asthma by Targeting Janus Kinase 3-Dependent Leukotriene Synthesis in Mast Cells with 4-(3',5'-Dibromo-4'-hydroxyphenyl)amino-6,7-dimethoxyquinazoline (WHI-P97)

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ABSTRACT

4-(3',5'-Dibromo-4'-hydroxyphenyl)amino-6,7-dimethoxyquinazoline (WHI-P97) is a rationally designed potent inhibitor of Janus kinase (JAK)-3. Treatment of mast cells with WHI-P97 inhibited the translocation of 5-lipoxygenase (5-LO) from the nucleoplasm to the nuclear membrane and consequently 5-LO-dependent leukotriene (LT) synthesis after IgE receptor/FceRI crosslinking by >90% at low micromolar concentrations. WHI-P97 did not directly inhibit the enzymatic activity of 5-LO, but prevented its translocation to the nuclear membrane without affecting the requisite calcium signal. WHI-P97 was very well tolerated in mice, with no signs of toxicity at dose levels ranging from 5 μg/kg to 50 mg/kg, and LD₁₀ was not reached at a 50 mg/kg dose level when administered as a single i.p. or i.v. bolus dose.

Therapeutic WHI-P97 concentrations, which inhibit mast cell leukotriene synthesis in vitro, could easily be achieved in vivo after the i.v. or i.p. administration of a single nontoxic 40 mg/kg bolus dose of WHI-P97. Notably, WHI-P97 showed promising biological activity in a mouse model of allergic asthma at nontoxic dose levels. Treatment of ovalbumin-sensitized mice with WHI-P97 prevented the development of airway hyper-responsiveness to methacholine in a dose-dependent fashion. Furthermore, WHI-P97 inhibited the eosinophil recruitment to the airway lumen after the ovalbumin challenge in a dose-dependent fashion. Further development of WHI-P97 may therefore provide the basis for new and effective treatment as well as prevention programs for allergic asthma in clinical settings.

Mast cells participate in the pathophysiology of allergy and asthma through the release of chemical mediators, including pro-inflammatory leukotrienes (LTs) after crosslinking of their high affinity surface IgE receptors/FceRI (Ishizaka et al., 1971; Wasserman, 1990; Galli, 1993; Malaviya et al., 1993). LT synthesis in mast cells is triggered by activation of the 5-lipoxygenase (5-LO) pathway (Jakschik and Lee, 1980). As a first step in this multistep process, the monooxygenase activity of 5-LO results in oxygenation of the 20-carbon fatty acid arachidonic acid to form 5-hydroperoxyeicosatetraenoic acid (5-HPETE). Next, the dehydrase activity of 5-LO catalyzes the conversion of 5-HPETE to an unstable epoxide intermediate (LTA₄), which is either converted by a zinc-dependent cytosolic hydrolase to leukotriene B₄ (LTB₄) or conjugated by a glutathione S-transferase (viz., LTC₄ synthesis) to glutathione to form the C6-peptide leukotriene C₄ (LTC₄) (An and Goetzl, 1998). LTB₄ as a potent chemotactic peptide can initiate a local inflammatory response by recruiting neutrophils (Zhang et al., 1992; Spada et al., 1997) and eosinophils (Henderson et al., 1996; Spada et al., 1997; Takeda et al., 1997). LTC₄ is converted to the other C6-peptide leukotrienes LTD₄ and LTE₄ (An, 1998). The C6-leukotrienes LTC₄, LTD₄, and LTE₄, as potent smooth muscle contractiles and vasoactive factors comprising the slow-reacting substance of anaphylaxis, participate in the pathophysiology of reactive airway disease and asthma by 1) inducing contractions of the airway smooth muscles (Drazen et al., 1980; Dahlen et al., 1983; Arm and Lee, 1992; Sorkness, 1997; Drazen et al., 1997), thereby increasing microvascular permeability and contributing to edema formation in the bronchial wall (Ramos et al., 1992), both of which lead to bronchoconstriction, and 2) stimulating mucus secretion.

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ABBREVIATIONS: LT, leukotriene; WHI-P97, 4-(3',5'-dibromo-4'-hydroxyphenyl)amino-6,7-dimethoxyquinazoline; WHI-P131, 4-(4'-hydroxyphenyl)amino-6,7-dimethoxyquinazoline; WHI-P197, 4-(3',5'-dibromo-4'-hydroxyphenyl)amino-6,7-dimethoxyquinazoline; WHI-P112, 4-(2',5'-dibromophenyl)amino-6,7-dimethoxyquinazoline; 5-LO, 5-lipoxygenase; FLAP, 5-LO-activating protein, 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; TX, thromboxane; PG, prostaglandin; PMSF, phenylmethylsulfonyl fluoride; JAK3, Janus kinase 3; DNP, dinitrophenyl; BMMC, bone marrow mast cells; TEA, triethylamine; ELISA, enzyme-linked immunosorbent assay; OVA, ovalbumin; Penh, enhanced pause; COX, cyclooxygenase; PIPES, 1,4-piperazinediethanesulfonic acid.
tion in the airways (Henderson et al., 1996), which can aggravate the airway obstruction. Furthermore, LTD₄ is selectively chemotactic for eosinophils (Spada et al., 1994) and LTE₄ may also promote eosinophil chemotaxis (Laitinen et al., 1993). The 5-LO is an 80-kDa protein constitutively expressed in nucleus as well as in the cytosol of the mast cells (Jakschik and Lee, 1980; Malaviya et al., 1993; Peters-Golden, 1998), and it can be activated by calcium and ATP (Jakschik and Lee, 1980). Recent studies have shown that stimulation of mast cells with a calcium ionophore results in the translocation of 5-LO to the nuclear envelope where it interacts with its substrate (Brock et al., 1998) to produce leukotrienes and is subsequently irreversibly inactivated (Wong et al., 1992; Malaviya et al., 1993). The interaction of 5-LO with substrate, arachidonic acid, is facilitated by 5-lipoxygenase-activating protein (FLAP), which is thought to act as an arachidonic acid-presenting protein (Abramovitz et al., 1993). By comparison, the membrane translocation of 5-LO after IgE receptor/FcεRI engagement in mast cells is not followed by an irreversible inactivation step (Malaviya et al., 1993). Consequently, 5-LO remains active and the capacity of IgE-sensitized mast cells to release leukotrienes is retained after subsequent antigen challenge (Malaviya et al., 1993). Later similar observations were made by Brock et al. (1998) with ionophore stimulation if the cells were stimulated with shorter time points. Therefore, repeated stimulation of mast cells in patients with allergic asthma may cause sustained synthesis and release of leukotrienes contributing to the significant and persistent bronchoconstriction and inflammatory airway response during episodes of exacerbation. In recent years, several strategies aimed at inhibiting the synthesis and release of leukotrienes (e.g., use of 5-LO inhibitors) or blocking their action at the receptor level (e.g., use of specific LTD₄ antagonists) have been explored as treatment modalities for asthma (Gorene et al., 1994; Smith, 1996; Sorkness, 1997; Tan, 1998).

Janus kinase 3 (JAK3), a member of the Janus family protein tyrosine kinases, is abundantly expressed in mast cells and plays a pivotal role in IgE receptor-mediated mast cell responses (Malaviya and Uckun, 1999; Malaviya et al., 1999). Recently, we used a novel homology model of the kinase domain of JAK3 for structure-based design of dimesoxyquinazoline compounds with potent and specific inhibitory activity against JAK3 (Sudbeck et al., 1999). Because as yet unidentified tyrosine kinases have been implicated in the catalysis and translocation of 5-LO (Lepley et al., 1996) we set out to determine whether a specific JAK3 inhibitor could be useful as a modulator of mast cell LT synthesis and/or release. The purpose of the present study was to examine the in vitro effects of our lead compound 4-(3',5'-dibromo-4'-hydroxyphenyl)amino-6,7-dimethoxynquinazoline (WHI-P97) (Sudbeck et al., 1999) on 5-LO-dependent leukotriene release from mast cells after IgE receptor crosslinking and to determine its in vivo biological activity in a mouse model of allergic asthma.

Here, we report that treatment of mast cells with WHI-P97 inhibits the membrane translocation of 5-LO and consequently 5-LO-dependent LT synthesis after IgE receptor/FcεRI crosslinking by >90% at low micromolar concentrations. WHI-P97 does not directly inhibit the enzymatic activity of 5-LO, but prevents its translocation from the nucleoplasm to the nuclear membrane. WHI-P97 was very well tolerated in mice with no signs of toxicity at dose levels ranging from 5 μg/kg to 50 mg/kg, and therapeutic WHI-P97 concentrations, which inhibit mast cell leukotriene synthesis in vitro, could easily be achieved in vivo after the i.v. or i.p. administration of a single nontoxic 40 mg/kg bolus dose of WHI-P97. Notably, WHI-P97 showed promising biological activity in a mouse model of allergic asthma at nontoxic dose levels. Treatment of OVA-sensitized mice with WHI-P97 prevented the development of airway hyper-responsiveness to methacholine in a dose-dependent fashion. Furthermore, WHI-P97 inhibited the eosinophil recruitment to the airway lumen after the OVA challenge in a dose-dependent fashion. Further development of WHI-P97 may therefore provide the basis for new and effective treatment as well as prevention programs for allergic asthma in clinical settings.

Materials and Methods

Mice. BALB/c mice (both sexes), SCID, C57BL/6, and female CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed in a controlled environment (12-h light/12-h dark photoperiod, 22 ± 1°C, 60 ± 10% relative humidity), which is fully accredited by the United States Department of Agriculture. Breeder pairs of JAK3-null mice were obtained from Dr. J. Ihle (St. Jude Children’s Research Hospital, Memphis, TN). All mice were housed in groups of five in microisolation cages (Lab Products, Inc., Maywood, NY) containing autoclaved bedding. Mice were provided free access to autoclaved pellet food and tap water. Animal studies were approved by the Hughes Institute Animal Care and Use Committee, and all animal care procedures conformed to the Principles of Laboratory Animal Care (National Institutes of Health Publication 85-23, revised 1985).

Chemicals and Reagents. The chemical synthesis and characterization of the JAK3 inhibitors WHI-P97 and WHI-P131 (4′-4′-hydroxyphenyl)amino-6,7-dimethoxynquinazoline) and the structurally similar control compound WHI-P112 (4′-2′,5′-dibromophenylamino-6,7-dimethoxynquinazoline), which does not inhibit JAK3, have been described elsewhere (Malaviya et al., 1999; Sudbeck et al., 1999) (Fig. 1A). The calcium ionophore A23187, cysteine, soybean trypsin inhibitor, phenylmethylsulphonyl fluoride (PMSF), bovine serum albumin, bromphenol blue, Tween 20, glycerol, and methacholine were purchased from Sigma Chemical Co. (St. Louis, MO). 15-HPETE was obtained from Biomol Research Laboratories (Plymouth Meeting, PA) and polyvinylidene difluoride membranes were obtained from Millipore Corp. (Bedford, MA). Anti-rabbit IgG antibody coupled to horseshadish peroxidase and reagents for enhanced chemiluminescence were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). We obtained HPLC-grade methanol from Baxter (Muskegon, MI), BCA protein assay reagents from Pierce (Rockford, IL), arachidonic acid from NuChek Preparations Inc. (Elysian, MN), and prostaglandin (PG)B₂, 5-hydroxyeicosatetraenoic acid (5-HETE), assay kits for LTC₄, B₄, and thromboxane (TX)B₂ from Cayman Chemicals (Ann Arbor, MI). The preparation and characterization of dinitrophenyl (DNP)-BSA (Wei et al., 1986), monoclonal DNP-IgE (Liu et al., 1980; Leng et al., 1988), and monoclonal antibodies to 5-LO have been previously described (Malaviya et al., 1993).

RBL-2H3 Mast Cell Line and Mast Cell Cultures. RBL-2H3 cells were supplied by Dr. R. P. Siraganian (Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health). This adherent mucosal mast cell line expresses 10⁹ to 10⁹ high affinity IgE receptors/FcεRI per cell. RBL-2H3 cells serve as a convenient model to study mast cell functions (Wong et al., 1992; Hamawy and Siraganian, 1997). RBL-2H3 cells were maintained as monolayers in 175-cm² culture flasks in Eagle’s minimum essential medium with Earle’s salts (without L-glutamine)
supplemented with 20% fetal bovine serum and 2 mM L-glutamine (Wong et al., 1992).

**Mouse Mast Cell Culture.** Mast cells were cultured from the bone marrow specimens of JAK3-null (Jak3<sup>2/2</sup>) and Jak3<sup>1/1</sup> control mice in a medium supplemented with 25% WEHI-3 cell supernatant for 3 weeks, as previously described (Malaviya and Abraham, 1995; Malaviya and Uckun, 1999).

**Stimulation of Mast Cells and Mediator Assays.** RBL-2H3 cells and Jak3<sup>2/2</sup> and Jak3<sup>1/1</sup> bone marrow mast cells (BMMC) were sensitized with a monoclonal anti-DNP IgE antibody (0.24 mg/ml) for 1 h at 37°C in a 48-well tissue culture plate. Unbound IgE was removed by washing the cells with phosphate-buffered saline, pH 7.4. After washing, the BMMC were resuspended in RPMI HEPES buffer, whereas PIPES-buffered saline containing 1 mM calcium chloride was added to the monolayers of the RBL-2H3 cells. To study the biological effects of the test compounds, sensitized mast cells were further incubated with the test compounds at the indicated concentrations (or vehicle alone) for 1 h. The cells were then challenged with 20 ng/ml DNP-BSA for 30 min at 37°C. The plates were centrifuged at 200 g for 10 min at 4°C. Supernatants were removed and saved. LTC<sub>4</sub>, LTB<sub>4</sub>, and TXB<sub>2</sub> levels were also determined in cell free supernatants (Xu et al., 1993; Malaviya and Abraham, 1995). In some experiments, we examined the effects of the test compounds on LTC<sub>4</sub> release from RBL-2H3 cells stimulated with IgE/antigen in the presence of 20 μM arachidonic acid to bypass the phospholipase A<sub>2</sub>- and phospholipase D-dependent pathways of arachidonic acid production.

**5-Lipoxygenase Assays.** Monolayers of RBL-2H3 mast cells (in 6-well tissue culture plates) were washed with cold PBS and resuspended in homogenization buffer (35 mM sodium phosphate buffer, pH 7.4, 6 mM EDTA, 0.5 mM PMSF, 1 mM dithiothreitol, and 60 μg/ml soybean trypsin inhibitor). The cells were sonicated twice (350 Sonifier; Branson sonic Power Co., Danbury, CT) for 5 s in an ice bath. The homogenate was centrifuged at 4°C at 10,000g for 20 min. The supernatant obtained in the 10,000g centrifugation was further centrifuged at 100,000g for 60 min. 5-LO activity was assessed in 100,000g supernatants as described (Malaviya et al., 1993). The supernatants, 50–60 μg of protein/ml were incubated with 30 μM arachidonic acid, 6.5 mM calcium, 2 mM ATP, 5 μM 15-HPETE, 0.1 M Tris buffer, pH 7.0, and 6 mM EDTA. PGB<sub>2</sub> was added to each sample as an internal standard before processing. The samples were applied to C18 extraction columns (J. T. Baker Chemical Co., Phillipsburg, NJ) and eluted with 1 ml of methanol. Reversed phase HPLC (flow rate: 1 ml/min) was performed on a Synchropak C18 column (6.5-μm particle size) with a diode array detector (Hewlett Packard, Wilmington, DE) with methanol/water/acetic acid (72:28:0.05, v/v), pH 4.8 (adjusted with ammonium hydroxide) (solvent A), as the mobile phase. The polarity of the mobile phase was decreased by a gradient of methanol (solvent B) from 0 to 60% between 12 and 30 min. The 5-LO products were identified according to their comigration with standards as well as their specific UV spectra and quantitated by integration of the HPLC peaks. All 5-LO products were eluted by 40 min.

**Protein Assays.** The protein amounts in the various subcellular fractions were determined using the BCA protein assay kit (Pierce, Rockford, IL). A standard curve obtained from the BSA protein standard provided with the kit was used to calculate the protein concentration in each unknown sample.

**Western Blot Analysis of 5-Lipoxygenase Protein.** Unstimulated control and stimulated RBL-2H3 cells were quickly scraped off

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**Fig. 1.** A, chemical structures and chromatograms of WHI-P97 and WHI-P112 (internal standard). The chemical structure of WHI-P97 (A.1), WHI-P131 (A.2), and WHI-P112 (A.3) and representative chromatograms of A.1 and A.3 from (B.1) blank plasma and (B.2) plasma samples 10 min after i.p. injection of 40 mg/kg WHI-P97 to BALB/c mice are presented. I.S., internal standard.
the 6-well plates and pelleted by centrifugation for 20 s in a microcentrifuge (Beckman-Coulter, Ontario, CA). The cell pellets were snap-frozen, and the supernatants were analyzed for LTC_4 by immunoassay. The cell pellets were resuspended in 35 mM sodium phosphate buffer, pH 7.4, 2 mM EDTA, 0.5 mM PMSF, 1 mM dithiothreitol, and 60 µg/ml soybean trypsin inhibitor and homogenized. The homogenate was centrifuged at 4°C and 10,000g for 20 min. The supernatant obtained in the 10,000g centrifugation was further centrifuged at 100,000g for 60 min. The pellet obtained after this 100,000g spin was rinsed and resuspended by sonication in the homogenization buffer using a volume identical to that of the 100,000g supernatant. The 100,000g supernatant contained cytosolic as well as nucleoplasmatic fractions, whereas the 100,000g pellet fraction contained microsomal as well as nuclear membrane fractions (Brock and Peters-Golden, 1995). The presence of nucleoplasm in soluble fraction and nuclear membranes in particulate fraction was confirmed by the Western blot analysis of 100,000g supernatants and pellets by using antibodies for the nucleoplasm marker, topoisomerase I, and nuclear membrane marker LAP2 (Negri et al., 1992; Zini et al., 1994; Buendia et al., 1999). The 100,000g supernatant and pellets were mixed separately with sample buffer to obtain a final concentration of 10 mM Tris, pH 6.8, 2% SDS, 5% glycerol, and 60 µg/ml soybean trypsin inhibitor and homogenized. Sensitized RBL-2H3 cells were then treated with 300 µg/ml DNP-IgE. For determination of WHI-P97 Plasma Levels by HPLC Analysis, Stock solutions of WHI-P97 and WHI-P112 were prepared in methanol and stored at −20°C. These stock solutions were further diluted with 50% methanol to yield the appropriate working solutions for the preparation of the calibration standards. Acetonitrile/water containing 0.1% of trifluoroacetic acid and 0.1% triethylamine (TEA) (40:60, v/v) was used as the mobile phase for separating WHI-P97 from its internal standard, WHI-P112. The mobile phase was degassed automatically using an electronic degasser system. The analytical column was equilibrated, and HPLC was run under isocratic conditions using a flow rate of 1.0 ml/min at ambient temperature. The wavelength of detection was set at 254 nm. Peak width, response time, and slit width were set at >0.03 min, 0.5 s, and 8 nm, respectively. TEA has been demonstrated to be an important modifier for most of nitrogen-containing compounds, including quinoline derivatives (Chen et al., 1999a–d). Presence of TEA in the described mobile phase shortens the retention time and sharpens the peak for the compound WHI-P97. The retention times for WHI-P97 and for WHI-P112 (a structurally similar dimesoxyquinazoline derivative, which was used as an internal standard) were 4.8 and 7.1 min, respectively. At the retention times of the WHI-P97 and WHI-P112, no interfering peaks were observed in the blank plasma (Fig. 1B).

For determination of the plasma WHI-P97 levels, 5 µl of the internal standard WHI-P112 (25 µM) was added to a 50-µl plasma sample. For extraction, 7 ml of chloroform was then added to the plasma sample, and the mixture was vortexed thoroughly for 3 min. Following centrifugation (300g, 5 min), the aequorin layer was frozen using acetone/dry ice, and the organic phase was transferred into a clean test tube. The chloroform extracts were dried under a slow steady stream of nitrogen. The extract was reconstituted in 100 µl of methanol:water (9:1, v/v), and a 50-µl aliquot of this solution was used for HPLC analysis using a recently reported HPLC system (Chen et al., 1999a,b). With the described extraction conditions, the extraction recoveries from plasma were 89.2 ± 1.4% (range, 79.6–94.4%; N = 12) for WHI-P97 and 87.2 ± 1.8% (range, 76.8–95.9%; N = 12) for WHI-P112. To generate a standard curve, WHI-P97 was added to plasma to yield final concentrations of 0.25, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250, and 500 µM. Subsequently, 5 µl of the internal standard (WHI-P112, 25 µM) was added to each sample. The plasma samples with known amounts of WHI-P97 and its internal standard WHI-P112 were extracted as previously described, and the standard curves were generated by plotting the peak area ratios (WHI-P97/WHI-P112) against the drug concentrations tested. Unweighted linear regression analysis of the standard curves was performed using the CA-Cricket Graph III computer program, Version 1.0 (Computer Association, Inc., Islandia, NY). The standard curve was linear over the concentration-dose ranges tested and could be described by the regression equation: 

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\frac{Y}{X} = 6.13X + 0.05 (r > 0.999) \text{ for the plasma levels less than 10 µM, and } Y = 5.08X + 1.77 (r > 0.999) \text{ for the plasma levels greater than 10 µM, where } Y \text{ in µM is the agent recovered in plasma, and } X \text{ is the peak area ratio (WHI-P97/WHI-P112). The lowest limit of detection of WHI-P97 in 50 µl of plasma was 0.1 µM at the signal-to-noise ratio of } \sim 3.
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Pharmacokinetics in Mice. In pharmacokinetic studies, either female CD-1 or male BALB/c mice were injected either i.v. via the tail vein or i.p. with a 40 mg/kg bolus dose of WHI-97. Under methoxyflurane anesthesia, blood samples (−200 μl) were obtained from the ocular venousplexus by retroorbital venipuncture at 0, 3 (only in i.p.), 5, 10 (only in i.p.), 15, 30, and 45 min and 1, 2, 4, and 6 h after administration of WHI-P97. For studying the linearity of pharmacokinetics, we also collected the plasma samples at 10 min and 1 h after i.p. injection doses of 4, 12 (only in BALB/c), 40, 80, and 120 mg/kg WHI-P97. All collected blood samples were heparinized and centrifuged at 7000g for 5 min in a microcentrifuge to obtain plasma. The plasma samples were stored at −20°C until analysis. Aliquots of plasma were used for extraction and HPLC analysis, as described above.

Pharmacokinetic Modeling and Statistical Analysis. Pharmacokinetic modeling and pharmacokinetic parameter estimation were carried out using the pharmacokinetics software, WinNonlin Program, Version 2.1 (Pharsight, Mountain View, CA) (Chen et al., 1999c,d). An appropriate pharmacokinetic model was chosen on the basis of the lowest sum of weighted squared residuals, lowest Schwartz criterion, lowest Akaike's Information Criterion value, lowest standard errors of the fitted parameters, and dispersion of the residuals. The elimination half-life was estimated by linear regression analysis of the terminal phase of the plasma concentration time curves. The area under the concentration-time curve (AUC) was calculated by the trapezoidal rule between the first (0 h) and last sampling times plus C/k, where C is the concentration of last sampling and k is the elimination rate constant. Systemic clearance (CL) was determined by dividing the dose by the AUC. Bioavailability (F) was estimated using the equation F(%) = AUCi.v. - dosei.v./AUCi.p. - dosei.p.. Statistical analysis was performed using the Instat program, v.3.0 (GraphPad Software, San Diego, CA). The significance of differences between pharmacokinetic parameter values was analyzed using a two-tailed t test, and P values < 0.05 were considered significant.

In Vivo Leukotriene C4 Release. To examine the effect of WHI-P97 on LT release in vivo, BALB/c mice were injected i.v. with 1 μg of DNP-IgE in a 200-μl volume. After 24 h, mice were treated with WHI-P97 (40 mg/kg, i.p.). Control mice were treated with an equal volume of vehicle. Thirty minutes after the injection of WHI-P97 or vehicle, mice were challenged with 5 μg of antigen (DNP-BSA) i.p. in 200 μl of PBS containing 1 mM cysteine, pH 7.2. Mice were sacrificed by cervical dislocation 15 min after the antigen challenge. Peritoneal cavities of the mice were lavaged with 2 ml of ice-cold PBS. Lavage fluids were centrifuged at 4°C for 5 min at 300g. LTC4 levels were quantitated in cell-free peritoneal lavage samples by enzyme-linked immunosorbent assay (ELISA) (Malaviya and Abraham, 1995).

In other experiments, we examined the effect of the JAK3 inhibitors WHI-P97 on A23187-induced LTC4 release from RBL-2H3 cells in vivo. In brief, SCID mice were subjected to total body irradiation with 250 rads using a cesium irradiator, and each mouse was injected with an i.p. inoculum of 10 × 10⁶ RBL-2H3 cells (Uckun et al., 1992, 1993). Twenty-four hours later, mice were injected with 100 μg/kg A23187 in PBS containing 1 mM cysteine, pH 7.2, to induce leukotriene release from RBL-2H3 cells. To examine the effect of WHI-P97, mice were treated with 40 mg/kg WHI-P97 or vehicle (500 μl) i.p. 30 min before A23187 challenge. Fifteen minutes after the A23187 challenge, mice were sacrificed, and their peritoneal cavities were gently lavaged with 2 ml of ice-cold PBS containing 1 mM cysteine to harvest the RBL-2H3 cells. Lavage fluids were centrifuged at 4°C for 5 min at 300g to pellet RBL-2H3 cells representing morphologically >90% of the harvested cell population and SCID mouse peritoneal macrophages/mast cells and LTC4 levels in cell free peritoneal lavage samples were quantitated by ELISA (Malaviya and Abraham, 1995).

Mouse Model of Allergic Asthma. To examine the effect of the JAK3 inhibitors WHI-P97 or WHI-P131 on allergic asthma in mice, BALB/c mice were injected i.p. with 20 μg of ovalbumin (OVA) in alum on days 0 and 14. On days 21, 22, and 23, mice were challenged for 5 min with 2% OVA via their airways by ultrasonic nebulization (Henderson et al., 1996; Hamelmann et al., 1997a,b,c). Mice were assessed for “airway responsiveness” on day 24, as previously reported in detail (Hamelmann et al., 1997d; Lee et al., 1997) and briefly described below. In some experiments, allergic asthma was induced in JAK3+/− or JAK3−/− mice as described above. On day 23, mice were sacrificed, and their lungs were fixed in 10% buffered formalin under constant pressure. After routine paraffin embedding, 5-μm sections were stained with H&E or mucicarmine. The sections were examined under light microscope.

Determination of Airway Responsiveness. Airway responsiveness was measured in unrestrained mice by noninvasive whole body plethysmography using a BioSystem plethysmography instrument (BUXCO, Tolu, NY) (Hamelmann et al., 1997e; Lee et al., 1997). The chamber pressure was measured with a transducer connected to a preamplifier module and analyzed by system XA software (Lee et al., 1997). The chamber pressure was used as a measure of the difference between thoracic volume expansion or contraction and air volume removed or added to the chamber during breathing. The differential of this function with respect to time produced a pseudo-flow value that is proportionate to the difference between the rate of the thoracic volume expansion and nasal air flow. The pulmonary airflow obstruction assessed by measuring “Enhanced Pause” (Penh) using the following formula according to the manufacturer’s recommendations: Penh = PEP/PIP × Pause. Pehn reflects changes in the wave form of the chamber pressure signal from both inspiration (PIP) and expiration (PEP) and combines it with the timing comparison of early and late expiration (Pause). To measure the methacholine responses, mice were placed in the chamber and baseline readings were taken and averaged for 3 min. Mice were nebulized with saline or methacholine at increasing doses (1–100 mg/ml) for 3 min, and the Penh readings were taken and averaged for 3 min after each nebulization. To study the effect of JAK3 inhibitors on allergic asthma, mice were treated with WHI-P97 or WHI-P131 2 h before and 2 h after the OVA challenge on day 23.

Assessment of Eosinophil Infiltration. After airway responsiveness measurements, lungs were lavaged thoroughly with 1 ml of saline. The lavage fluid was centrifuged, and the supernatant was removed. The cell pellet was resuspended in saline containing 0.1% BSA to yield a final cell concentration of 0.1 × 10⁶/ml. Cytospin smears made from the cell suspension were stained with Diff-Quick, and the number of eosinophils was determined.

Results

Effects of JAK3 Inhibition with WHI-P97 or WHI-P131 on IgE Receptor/FceRI-Mediated Release of Ara-chidonic Acid-Derived Lipid Mediators from Mast Cells. Incubation of IgE-sensitized RBL-2H3 mast cells with the specific antigen DNP-BSA for 30 min caused them to release significant amounts of the leukotriene products of the 5-LO pathway, LTC4 (25.7 ± 5.6 ng/10⁶ cells) (Fig. 2A) and LTB4 (73 ± 12 pg/10⁶ cells) (Fig. 2B). A 1-h exposure of IgE-sensitized mast cells to the JAK3 inhibitor WHI-P97 (but not the structurally similar control dimethoxyquinazoline compound WHI-P112) before the antigen challenge reduced the released amounts of these LTs after antigen challenge in a concentration-dependent fashion (Fig. 2, A and B). The average IC₅₀ values were 6.8 μM for LTC₄ release and 21.0 μM for LTB₄ release. These results indicated that the WHI-P97 target JAK3 plays a key role in IgE receptor/FceRI-mediated LT (especially LTC₄) release from mast cells. We next compared the magnitude of LTC₄ release from bone marrow mast cells of wild-type mice to the magnitude of LTC₄ release from JAK3−/− bone marrow mast cells of JAK3−/− mice. The statistical analysis showed the significance of differences between the magnitudes of LTC₄ release from wild-type and JAK3−/− bone marrow mast cells.
knockout mice in three independent experiments. As shown in Fig. 2C, IgE-sensitized JAK3−/− mast cells released significantly less LTC4 than IgE-sensitized JAK3+/+ mast cells upon DNP-BSA challenge. These results provide direct experimental evidence that JAK3 is an important regulator of LT release from mast cells after IgE receptor/FcεRI crosslinking. Treatment of JAK3−/− mast cells with the JAK3 inhibitor WHI-P97 reduced their LTC4 release to the level of LTC4 release from JAK3+/+ mast cells (Fig. 2C). Treatment of JAK3+/+ mast cells with WHI-P97 did not result in further reduction in the amount of LTC4 release upon IgE receptor/FcεRI crosslinking (LTC4 release: no WHI-P97, 7.2 ± 2.6 versus +WHI-P97, 5.4 ± 1.1; P = 0.2). Thus, the WHI-P97-caused inhibition of LTC4 release from JAK3+/+ mast cells was not due to promiscuous inhibition of other enzymes by this JAK3 inhibitor.

The LT synthesis inhibitory activity of WHI-P97 was also compared with a previously reported JAK3 inhibitor, WHI-P131 (Malaviya et al., 1999). Similar to WHI-P97, WHI-P131 also inhibited the IgE/FcεRI-receptor-mediated mast cells LTC4 release in a concentration-dependent fashion (Fig. 1A). The inhibitory effect on LT release was not due to reduced cell viability, because >95% of mast cells remained capable of

![Graph A](image1.png)

**Fig. 2.** Effect of WHI-P97 on IgE receptor/FcεRI-mediated mediator release in mast cells. Anti DNP-IgE-sensitized RBL-2H3 cells were incubated with indicated concentrations of the test compounds for 1 h before challenge with 20 ng/ml antigen (DNP-BSA). LTC4 (A) and LTB4 (B) formation was measured in cell-free supernatants by immunoassay. The data points represent the mean ± S.E. values obtained from three to five experiments. *P < .05 compared with control as determined by Student's t test. C, IgE sensitized BMMC cells from JAK3+/+ and Jak3−/− mice were incubated with 10 μM WHI-P97 or vehicle for 15 min and then challenged with DNP-BSA as described in detail under Materials and Methods. LTC4 levels were estimated in cell-free supernatants of BMMC. The data points represent the mean ± S.E. values (n = 3). *P < .05 compared with the vehicle-treated control as determined by Student's t test.

![Graph B](image2.png)

**Fig. 3.** Effect of WHI-P97 on IgE receptor/FcεRI-mediated mast cell TXB2 release and arachidonic acid metabolism to LTC4. Anti-DNP-IgE-sensitized RBL-2H3 cells were incubated with indicated concentrations of WHI-P97 and stimulated with DNP-BSA as described in Fig. 2. Mast cell TXB2 (A) formation was measured in cell-free supernatants using an immunoassay. The results are expressed as percentage of vehicle-treated controls. To show that the inhibition of leukotriene synthesis by WHI-P97 was not due to its effect on phospholipase activity, IgE-sensitized RBL-2H3 cells were incubated with 20 μM arachidonic acid (AA) before antigen challenge, and LTC4 (B) release in extracellular medium was measured using an immunoassay. The results of LTC4 release are expressed as percentage of vehicle-treated controls. The data points represent the mean ± S.E. values obtained from three experiments. **P < .005 compared with the corresponding control as determined by Student's t test.
Trypan Blue dye exclusion after treatment with 100 μM WHI-P97 or WHI-P131 (data not shown). Similarly, the metabolic activity of the cyclooxygenase (COX) pathway, as measured by antigen-induced thromboxane (TX) B₂ release from IgE-sensitized mast cells (Xu et al., 1993), was not affected by WHI-P97 over a concentration range of 1–30 μM, and it was inhibited by 25% at 100 μM (Fig. 3A). The lack of a significant effect on the TXB₂ output of the arachidonic acid-dependent COX pathway also showed that the WHI-P97-induced inhibition of leukotriene synthesis in mast cells was not caused by a reduced release of arachidonic acid from the intracellular phospholipid sources due to inhibition of phospholipases (i.e., inhibition of phospholipase A₂, which cleaves arachidonic acid from phosphatidylcholine; inhibition of phospholipase C, which acts on phosphatidylinositol to generate diacylglycerol as a diacylglycerol lipase substrate and precursor of arachidonic acid; or inhibition of phospholipase D, which converts phosphatidylcholine to a precursor of diacylglycerol).

To further confirm the validity of this latter conclusion, we also examined the effects of the lead JAK3 inhibitor WHI-P97 on LTC₄ release from mast cells in the presence and absence of exogenous arachidonic acid. Sensitized mast cells were stimulated with antigen in the presence or absence of

![Fig. 4. Effect of WHI-P97 on mast cell 5-LO activity. IgE-sensitized RBL-2H3 cells were incubated with 30 μM WHI-P97 or vehicle for 30 min and then challenged with 20 ng/ml DNP-BSA or 1 μM A23187 for 15 min. Cell culture supernatants were analyzed for LTC₄ release (A) and 100,000 g supernatants of stimulated and unstimulated RBL-2H3 cells were analyzed for 5-LO activity (B). The LTC₄ release data are expressed as ng/10⁶ cells. The 5-LO activity is expressed as 5-HETE formation (ng/μg of protein). The data points represent the mean ± S.E. values obtained from three experiments. ND, not detectable. *P < .05 compared with the vehicle-treated control; **P < .005 compared with the WHI-P97-treated samples as determined by Student’s t test.](#)

![Fig. 5. Effect of WHI-P97 and WHI-P131 on A23187-induced 5-LO translocation in mast cells. RBL-2H3 cells were incubated with WHI-P97, WHI-P131, WHI-P112, or vehicle for 60 min before stimulation with 1 μM ionophore (A23187) for 15 min to initiate the translocation of 5-LO from cytosol and nucleoplasm to the nuclear envelope. A1 and A2, control 100,000 g supernatants (S) and membrane fractions (M) were subjected to Western blot analysis using antibodies for the nucleoplasm marker topoisomerase I and the nuclear membrane marker LAP2, as described under Materials and Methods. B and C, immunoblot analysis of 5-LO was performed in 100,000 g supernatants and membrane fractions. B, the bands on the blots were analyzed by densitometry, the amount of 5-LO present in pellets is expressed as the percentage of total (S + M), and the mean ± S.E. values were calculated (n = 3–4). C, a representative blot. *P < .05 compared with the A23187-stimulated and vehicle-treated control as determined by Student’s t test.](#)
Marachidonic acid as well as in the presence or absence of WHI-P97. Mast cells stimulated with IgE/antigen in the presence of arachidonic acid released four times more LTC\(_4\) (412 \pm 63\%) than mast cells stimulated in the absence of arachidonic acid (Fig. 3B). This result was anticipated because a significant increase in substrate (i.e., arachidonic acid) availability is likely to cause increased LTC\(_4\) synthesis (Coffey et al., 1994). Notably, WHI-P97 treatment resulted in marked inhibition of the LTC\(_4\) release from stimulated mast cells even in the presence of exogenous arachidonic acid (Fig. 3B). Furthermore, when we compared the release of LTC\(_4\) of WHI-P97-pretreated and IgE/antigen-stimulated mast cells in the presence and absence of arachidonic acid, as shown in Fig. 3B, we found an 8-fold increase in the LTC\(_4\) synthesis in the presence of arachidonic acid. These results are consistent with previous findings showing that high concentrations of arachidonic acid bypass the need of 5-LO translocation and FLAP requirement (Abramovitz et al., 1993; Coffey et al., 1994). These results also indicate that WHI-P97 likely inhibits the synthesis of LTC\(_4\) by 1) preventing the proximal signaling events leading to the activation of 5-LO, 2) directly inhibiting the enzymatic activity of 5-LO, or 3) blocking the translocation of 5-LO to the membrane.

20 \(\mu\)M arachidonic acid as well as in the presence or absence of WHI-P97. Mast cells stimulated with IgE/antigen in the presence of arachidonic acid released four times more LTC\(_4\) (412 \pm 63\%) than mast cells stimulated in the absence of arachidonic acid (Fig. 3B). This result was anticipated because a significant increase in substrate (i.e., arachidonic acid) availability is likely to cause increased LTC\(_4\) synthesis (Coffey et al., 1994). Notably, WHI-P97 treatment resulted in marked inhibition of the LTC\(_4\) release from stimulated mast cells even in the presence of exogenous arachidonic acid (Fig. 3B). Furthermore, when we compared the release of LTC\(_4\) of WHI-P97-pretreated and IgE/antigen-stimulated mast cells in the presence and absence of arachidonic acid, as shown in Fig. 3B, we found an 8-fold increase in the LTC\(_4\) synthesis in the presence of arachidonic acid. These results are consistent with previous findings showing that high concentrations of arachidonic acid bypass the need of 5-LO translocation and FLAP requirement (Abramovitz et al., 1993; Coffey et al., 1994). These results also indicate that WHI-P97 likely inhibits the synthesis of LTC\(_4\) by 1) preventing the proximal signaling events leading to the activation of 5-LO, 2) directly inhibiting the enzymatic activity of 5-LO, or 3) blocking the translocation of 5-LO to the membrane.

Effects of JAK3 Inhibition with WHI-P97 or WHI-P131 on the Enzymatic Activity and Membrane Translocation of 5-Lipooxygenase in Mast Cells after IgE Receptor Crosslinking or Calcium Ionophore (A23187) Treatment. The inhibition of LTC\(_4\) synthesis in mast cells after pretreatment with WHI-P97 could be due to inhibition of the enzymatic activity of 5-LO or its translocation to the membrane. We first investigated the effect of WHI-P97 on the enzymatic activity of 5-LO. The soluble 5-LO activity of IgE-sensitized RBL-2H3 cells, as measured by the cumulative amount of 5-HETE produced in the presence of 30 \(\mu\)M arachidonic acid during a 15-min incubation, ranged from 13.0 to 17.0 ng of 5-HETE/\(\mu\)g of protein in the absence of IgE receptor/FceRI crosslinking. In accord with our own observations (Malaviya et al., 1993) and those of others (Wong et al., 1992), stimulation of IgE-sensitized mast cells with antigen (20 ng/ml DNP-BSA) triggered LTC\(_4\) release (11.0 \pm 0.8 ng/10\(^6\) cells) (Fig. 4A) without significantly inhibiting the activity of 5-LO (Fig. 4B), whereas treatment with A23187 (1 \(\mu\)M) triggered LTC\(_4\) release (16.8 \pm 1.2 ng/10\(^6\) cells) (Fig. 4A), which was accompanied by inactivation of 5-LO (Fig. 4B). WHI-P97 (30 \(\mu\)M) substantially reduced the IgE/antigen-induced LTC\(_4\) release from mast cells (Fig. 4A) without in-
hibiting the enzyme activity of 5-LO (Fig. 4B). Indeed, WHI-P97 appeared to prevent the slight reduction of 5-LO activity after IgE receptor crosslinking (Fig. 4B). WHI-P97 (30 μM) also inhibited the LTC4 release after A23187 stimulation (Fig. 4A). Notably, WHI-P97 prevented the A23187-induced inactivation of 5-LO (Fig. 4B), which prompted the hypothesis that this compound may block the translocation of 5-LO from soluble form to the membrane-bound form after IgE receptor crosslinking or calcium ionophore treatment.

We next examined the effect of WHI-P97 on A23187-induced translocation of 5-LO in mast cells by Western Blot analysis. To this end, we isolated the 100,000 g supernatants (i.e., cytosolic as well as nucleoplasmic fractions) and pellets (i.e., microsomal as well as nuclear membrane fractions) from RBL-2H3 mast cells. These fractions were first subjected to Western blot analysis using antibodies for the nucleoplasm marker topoisomerase I and the nuclear membrane marker LAP2, as described under Materials and Methods. As anticipated, topoisomerase I was detected only in the soluble fraction, and LAP2 was detected only in the pellets (Fig. 5A).

![Fig. 7. Plasma concentration-time profiles of WHI-P97. Plasma concentration-time profiles of WHI-P97 after i.v. bolus injection (40 mg/kg; five mice per group) to CD-1 mice (A) and to BALB/c mice (B), and after i.p. administration (40 mg/kg; four mice per group) to CD-1 mice (C) and to BALB/c mice (D).](image)

**Table 1**

Pharmacokinetic parameter values of WHI-P97 in CD-1 mice and BALB/c mice

Pharmacokinetic parameter values in mice are present as the average values estimated from composite plasma concentration-time curves of pooled data. The mean ± S.E. values are indicated in parentheses.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Dose</th>
<th>Vc</th>
<th>Vss</th>
<th>CL</th>
<th>AUC</th>
<th>F</th>
<th>Cmax</th>
<th>t1/2</th>
<th>tmax</th>
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<tr>
<td></td>
<td></td>
<td>m/l kg</td>
<td>m/l kg</td>
<td>m/l h/kg</td>
<td>μM·h</td>
<td>%</td>
<td>μM</td>
<td>min</td>
<td>min</td>
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<tr>
<td>CD-1</td>
<td>i.v.</td>
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<td>322</td>
<td>431</td>
<td>891</td>
<td>107.3</td>
<td>N.D.</td>
<td>296.7</td>
<td>58.9</td>
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<td></td>
<td></td>
<td>(290 ± 28)</td>
<td>(488 ± 59)</td>
<td>(838 ± 71)</td>
<td>(116.9 ± 8.1)</td>
<td></td>
<td></td>
<td>(342.2 ± 32.1)</td>
<td>(105.1 ± 43.2)</td>
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<tr>
<td>BALB/c</td>
<td>i.v.</td>
<td>415</td>
<td>681</td>
<td>1513</td>
<td>58.4</td>
<td>N.D.</td>
<td>212.7</td>
<td>84.2</td>
<td>N.D.</td>
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<td></td>
<td></td>
<td>(417 ± 26)</td>
<td>(833 ± 59)</td>
<td>(1519 ± 46)</td>
<td>(58.3 ± 1.7)</td>
<td></td>
<td></td>
<td>(215.1 ± 13.1)</td>
<td>(134.6 ± 43.4)</td>
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<tr>
<td>CD-1</td>
<td>i.p.</td>
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<td>229</td>
<td>N.D.</td>
<td>5208</td>
<td>18.3</td>
<td>17.1</td>
<td>47.6</td>
<td>101.9</td>
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<td>(231 ± 41)</td>
<td>(4649 ± 224)</td>
<td>(20.7 ± 1.0)</td>
<td>(19.3 ± 0.8)</td>
<td>(147.9 ± 33.3)</td>
<td>(2.1 ± 0.5)</td>
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<tr>
<td>BALB/c</td>
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<td>507</td>
<td>N.D.</td>
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<td>27.7</td>
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<td>(285 ± 16)</td>
<td>(5531 ± 272)</td>
<td>(16.1 ± 0.8)</td>
<td>(27.6 ± 1.3)</td>
<td>(57.5 ± 9.5)</td>
<td>(85.6 ± 6.3)</td>
<td>(2.9 ± 1.0)</td>
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</tbody>
</table>

* a Apparent systemic clearance without correction by bioavailability (F).

* b t1/2, terminal elimination half-life; tmax, time required to reach the maximum plasma drug concentration (Cmax) following i.p. administration; N.D., no determination; Vc, apparent volume of distribution; Vss, apparent steady-state volume of distribution.
Western blot analysis of these fractions with an 5-LO antiserum (Malaviya et al., 1993) demonstrated that in unstimulated mast cells, the majority of the 5-LO protein resides in the soluble fraction (S). Treatment of mast cells with A23187 resulted in translocation of 5-LO to the membrane fraction (M) as evidenced by a significant increase in the amount of 5-LO found in the membrane fraction (Fig. 5, B and C) and a concomitant reduction in the amount of 5-LO remaining in the soluble fraction (Fig. 5C). WHI-P97 (but not the control compound WHI-P112) inhibited the translocation of 5-LO from the soluble fraction to the membranes in a concentration-dependent fashion (Fig. 5B). Similar to WHI-P97, WHI-P131 also inhibited the A23187-induced 5-LO translocation in mast cells (Fig. 5C).

Recent studies have shown that the majority of the cellular 5-LO in RBL-2H3 cells is localized in the nucleoplasm and, upon activation, 5-LO is translocated to the nuclear envelope (Brock et al., 1994, 1988; Coffey et al., 1994). We next used immunofluorescent confocal laser scanning microscopy to confirm that WHI-P97 inhibits the IgE/antigen-induced translocation of 5-LO from the nucleoplasm to the nuclear envelope of IgE-sensitized RBL-2H3 mast cells. In unstimulated control RBL-2H3 cells, the bulk of 5-LO was localized in the nucleoplasm and displayed a granular staining pattern (Fig. 6A), in accordance with previous reports (Brock et al., 1994, 1988). Upon stimulation with 20 ng/ml DNP-BSA, 5-LO was translocated to the nuclear envelope, as evidenced by the disappearance of the granular nucleoplasmic staining and appearance of a bright fluorescent rim around the nucleus (Fig. 6B), which is consistent with earlier reports (Brock et al., 1994, 1988; Coffey et al., 1994). The confocal images of WHI-P97 (30 μM)-pretreated RBL-2H3 cells depicted in Fig. 6C show that the bulk of 5-LO is still localized in the nucleoplasm, thereby providing direct and unambiguous evidence that WHI-P97 prevents the IgE/antigen-induced 5-LO translocation to the nuclear membrane. Similar results were obtained with the other JAK3 inhibitor WHI-P131 (Fig. 6D).

Pharmacokinetics of the JAK3 Inhibitor WHI-P97 in Mice. We have previously reported the favorable pharmacokinetics and toxicity profile of WHI-P131 (Chen et al., 1999b; Malaviya et al., 1999). WHI-P97 was also very well tolerated in mice with no signs of toxicity at dose levels ranging from 5 μg/kg to 50 mg/kg and LD$_{10}$ was not reached at a 50 mg/kg dose level when WHI-P97 was administered as a single i.p. or i.v. bolus dose. In particular, we observed no decrease in activity level of mice, weight loss, diarrhea, seizures, or death. There were no drug-related toxic lesions in any of the organs of the WHI-P97-treated mice that were electively sacrificed on day 30 (data not shown).

The plasma concentration-time curves of WHI-P97 in CD-1 and BALB/c mice after i.v. injection of a 40 mg/kg bolus dose are depicted in Fig. 7, A and B. A two-compartment, first order pharmacokinetic model was applied to analyze the plasma concentration-time curves. The pharmacokinetic parameter values are shown in Table 1. WHI-P97 had an elimination half-life ($t_{1/2}$) of 58.9 min and systemic clearance (CL) of 891 ml/h/kg in CD-1 mice and a $t_{1/2}$ of 84.2 min and CL of 1513 ml/h/kg in BALB/c mice. The values for AUC and $C_{\text{max}}$ were 107.3 μM · h and 296.7 μM, respectively, in CD-1 mice, and 58.4 μM · h and 212.7 μM, respectively, in BALB/c mice. The large volume of distribution [322 ml/kg in CD-1 mice and 415 ml/kg in BALB/c mice; ~6-fold greater than the plasma volume (50 ml/kg)] (Davies and Morris, 1993) suggests that WHI-P97 may be extensively partitioned into extravascular compartments.

In both CD-1 and BALB/c mice, a two-compartment, first order pharmacokinetic model was fit to the plasma concentration-time curves obtained after the i.p. injection of a single 40 mg/kg bolus dose of WHI-P97 (Fig. 7, C and D). The
computer-estimated pharmacokinetic parameter values are shown in Table 1. In CD-1 mice, the estimated maximum plasma concentration ($C_{\text{max}}$) of WHI-P97 after i.p. administration was 47.6 μM, and its bioavailability, $F$, was estimated to be 17.1%. WHI-P97 demonstrated rapid absorption after i.p. administration, and the estimated time to reach the maximum plasma WHI-P97 concentration ($t_{\text{max}}$) was only 2.8 min. WHI-P97 also had a moderately rapid elimination rate with an elimination half-life of 101.9 min. The central volume distribution of WHI-P97 was 229 ml/kg, indicating that this agent is extensively partitioned into extravascular compartments after i.p. administration. In BALB/c mice, the estimated values for $C_{\text{max}}$, $F$, $t_{\text{max}}$, and $V_c$ were 51.9 μM, 27.7%, 3.2 min, 82.5 min, and 307 ml/kg, respectively (Table 1).

At 10 min after i.p. injection doses of 4, 40, 80, and 120 mg/kg to CD-1 mice, the measured WHI-P97 levels (mean ± S.E.) were $10.2 ± 1.8, 20.2 ± 2.7, 38.9 ± 3.9, and 50.2 ± 2.2$ μM, respectively (Fig. 8A). These results are consistent with a linear dose-dependent pharmacokinetics pattern in CD-1 mice. In BALB/c mice, the plasma WHI-P97 levels at 10 min after administration were $6.7 ± 0.5, 23.6 ± 1.6, 24.7 ± 1.2, 23.5 ± 1.8, and 31.2 ± 2.2$ μM after i.p. injection of 4, 12, 40, 80, and 120 mg/kg of WHI-P97, respectively. These results indicate a nonlinear pharmacokinetic profile in BALB/c mice at dose levels higher than 12 mg/kg. Most importantly, WHI-P97 concentrations that inhibit mast cell leukotriene synthesis in vitro could easily be achieved and maintained for 3 to 4 h after the i.v. or i.p. administration of a single nontoxic 40 mg/kg bolus dose of WHI-P97.

Effect of the JAK3 Inhibitor WHI-P97 on LTC₄ Release in Vivo. Our pharmacodynamic studies prompted the

Fig. 9. Inhibition of LTC₄ release by WHI-P97 in mice. The effects of WHI-P97 on IgE/antigen (A)- and A23187 (B)-induced LTC₄ release in vivo was examined as described under Materials and Methods. A, peritoneal cells of IgE-sensitized mice were challenged with DNP-BSA (5 μg) for 15 min. To study the effect of WHI-P97 IgE/antigen-induced LTC₄ release, IgE-sensitized mice were injected with 40 mg/kg WHI-P97 or vehicle 30 min before the antigen challenge. B, irradiated SCID mice were injected with $10^5$ RBL-2H3. To examine the effect of WHI-P97 on in vivo LTC₄ release by RBL-2H3 cells, mice were treated with 40 mg/kg WHI-P97 i.p. 30 min before A23187 challenge. LTC₄ levels were quantitated in cell-free peritoneal lavage fluids by ELISA. The data points represent the mean ± S.E. values obtained in three to five mice. $^*P < .05$ compared with the IgE/Ag or A23187-stimulated control as determined by Student's $t$ test.

Fig. 10. Inhibition of airway responsiveness by WHI-P97 in mice. Mice were sensitized with OVA on days 0 and 14 i.p. as detailed under Materials and Methods. On days 20, 21, and 23, mice were challenged for 5 min with 2% OVA using a nebulizer. After 24 h, airway responsiveness to increasing doses of methacholine was measured. To study the effect of JAK3 inhibitors on methacholine-induced airway hyper-responsiveness, mice were injected i.p. with indicated doses of WHI-P97, WHI-P131, or vehicle 1 h before and 2 h after OVA challenge on day 23. The data points represent the mean ± S.E. values obtained in 8 to 13 mice.
hypothesis that biologically effective mast cell inhibitory plasma concentrations of WHI-P97 can be achieved in vivo at nontoxic (i.e., <50 mg/kg) dose levels. To test this hypothesis, we first studied the effects of a single nontoxic i.p. bolus dose of WHI-P97 on antigen-induced LTC₄ release in IgE-sensitized BALB/c mice, as described under Materials and Methods. Mice were sensitized with IgE by injecting them i.v. with 1 μg of DNP-IgE. IgE-sensitized BALB/c mice were treated prophylactically with WHI-P97 (40 mg/kg, i.p.) or vehicle 30 min before antigen challenge (5 μg of antigen DNP-BSA, i.p.), electively sacrificed 15 min after the antigen challenge, and their peritoneal cavities were lavaged with PBS. LTC₄ levels in these peritoneal lavage samples were determined by ELISA (Malaviya and Abraham, 1995). As shown in Fig. 9A, WHI-P97 inhibited IgE/antigen-induced LTC₄ release by 70%. We also examined the effect of WHI-P97 on A23187-induced in vivo LTC₄ release from RBL-2H3 cells previously inoculated into the peritoneal cavity of sublethally irradiated SCID mice, as described under Materials and Methods. Mice were treated prophylactically with i.p. injections of WHI-P97 (40 mg/kg) or vehicle 30 min before the A23187 challenge. As shown in Fig. 9B, WHI-P97 pretreatment resulted in 90% reduction of in vivo LTC₄ release from RBL-2H3 cells. Thus, WHI-P97 is a potent inhibitor of IgE/antigen-induced LTC₄ release from mouse peritoneal mast cells as well as A23187-induced LTC₄ release from the rat mast cell line RBL-2H3 in vivo.

Effect of the JAK3 Inhibitors WHI-P97 and WHI-P131 on Allergen-Induced Airway Hyper-Responsiveness.

Airway hyper-responsiveness is a cardinal feature of allergic asthma (Arm and Lee, 1992; Wasserman, 1994; Smith, 1996). We utilized a well characterized mouse model of allergic asthma to study the effects of the JAK3 inhibitors WHI-P97 and WHI-P131 on airway hyper-responsiveness using whole body plethysmography in nonrestrained conscious mice. In this model, sensitization of mice with OVA followed by airway challenge with the same antigen triggers the production of high levels of OVA-specific IgE and IgG1 (Hamelmann et al., 1997d). We compared the airway hyper-responsiveness of OVA-sensitized and saline-treated (PBS), OVA-sensitized/ OVA-challenged (OVA/OVA) mice, and WHI-P97- or WHI-P131-treated OVA-sensitized and challenged (OVA+WHI-P97) mice in bronchoprovocation tests with inhalation of methacholine. Mice treated with OVA showed significantly higher Penh in response to methacholine challenge as compared with control (PBS) mice (Fig. 10). In accordance with previous reports (Henderson et al., 1996; Hamelmann et al., 1997c), the methacholine dose required to induce a 100% increase of Penh in OVA-challenged mice was 3-fold lower than the methacholine dose required to induce a 100% increase of Penh in control mice (Fig. 10). Treatment of OVA-sensitized mice with increasing doses of WHI-P97 on day 24 prevented the development of airway hyper-responsiveness (Penh) in a dose-dependent fashion (Fig. 10A). Similar re-
sults were obtained with the other JAK3 inhibitor, WHI-P131 (Fig. 10B).

Effect of WHI-P97 on Eosinophil Infiltration. Airway hyper-responsiveness of asthmatic patients is maintained as a result of persistent airway inflammation. Eosinophils are the prominent cells involved in the airway inflammation of asthmatics and are found in large numbers in sputum and bronchoalveolar lavage fluids. LTs have been implicated in eosinophil recruitment and mucus release in allergic asthma (Henderson et al., 1996). We found that JAK3−/− mast cells cultured from the bone marrow cells of JAK3-knockout mice release markedly reduced amounts of LTC₄ than wild-type (JAK3+/+) mast cells cultured from bone marrow cells of JAK3−/− mice in response to IgE receptor crosslinking (Figs. 2C). Furthermore, compared with wild-type mice there was poor eosinophil recruitment and mucus secretion in JAK3-knockout mice (Fig. 11) to the airway lumen after OVA challenge. These findings prompted the hypothesis that a potent inhibitor of JAK3, such as WHI-P97 or WHI-P131, could prevent the eosinophil recruitment to the airway lumen after antigen challenge. To assess the effects of WHI-P97 and WHI-P131 on airway inflammation, we compared the eosinophil numbers in bronchoalveolar lavage samples of saline-challenged and OVA-challenged mice with or without WHI-P97/WHI-P131 treatment. As shown in Fig. 12A, significantly higher numbers of eosinophils were found in the airway lumen of mice challenged with OVA than in the airway lumen of mice challenged with saline. Pretreatment with WHI-P97 (Fig. 12A) or WHI-P131 (Fig. 12B) resulted in decreased eosinophil recruitment to the airway lumen after the OVA challenge in a dose-dependent fashion.

Discussion

Janus kinase 3 has traditionally been known to play an important role in lymphocyte development, activation, and cytokine responsiveness (Ihle and Kerr, 1995; Ihle et al., 1995; Sohn et al., 1998). We have recently discovered, using both JAK3-knockout mice and JAK3-specific tyrosine kinase inhibitors, that JAK3 also plays a pivotal role in IgE-receptor-mediated mast cell responses in vitro as well as in vivo (Malaviya and Uckun, 1999; Malaviya et al., 1999). Most importantly, JAK3 inhibitors effectively prevented cutaneous and systemic anaphylaxis in mice without causing any side effects (Malaviya and Uckun, 1999; Malaviya et al., 1999). We now show that the lead JAK3 inhibitor WHI-P97 is highly effective in a mouse model of allergic asthma at non-toxic dose levels.

Recent analyses of signal transduction events in mast cells have implicated JAK3 as a key regulator of IgE receptor/FccRI-mediated biochemical signals. Experimental evidence indicates that JAK3 and SYK cooperate in initiation of mast cell-mediated hypersensitivity reactions (Malaviya et al., 1999). It is well established that leukotrienes are produced by the metabolism of arachidonic acid after the activation of mitogen-activated protein kinases (Hirasawa et al., 1995; Costello et al., 1996). Using a JAK3 inhibitor (Malaviya et al., 1999), we have previously shown that JAK3 regulates IgE/antigen-induced mitogen-activated protein kinase activity in mast cells. The present study for the first time demonstrates that the JAK3 inhibitor WHI-P97 acts as a potent inhibitor of IgE receptor-mediated 5-LO-dependent leukotriene synthesis in mast cells without affecting the arachidonic acid-dependent COX pathway. WHI-P97 does not directly inhibit the enzymatic activity of 5-LO, but it prevents the IgE/antigen-induced translocation of 5-LO from the nucleoplasm/cytoplasm to the nuclear envelope. Similarly, WHI-P131, another known inhibitor of JAK3, also inhibited the membrane translocation of 5-LO and leukotriene synthesis in mast cells. These results uniquely indicate that JAK3 plays a critical role in IgE receptor-mediated leukotriene synthesis in mast
cells by regulating the activation of 5-LO after IgE receptor-crosslinking. Further studies will be needed to elucidate the exact molecular mechanism of WHI-P97-induced inhibition of 5-LO translocation and completely decipher the role of JAK3 in IgE receptor-mediated leukotriene synthesis.

In the present study, we discovered that exogenous arachidonic acid is capable of enhancing LTC4 synthesis by ~8-fold even in the presence of the JAK3 inhibitor WHI-P97. This finding indicates that LTC4 synthesis from exogenous arachidonic acid is less dependent on 5-LO translocation than the LTC4 synthesis from endogenous arachidonic acid. This finding is also consistent with previously published data showing that high concentrations of exogenous arachidonic acid can bypass the need for 5-LO translocation and for the arachidonic acid-presenting protein FLAP at the nuclear membrane to present endogenous arachidonic acid to 5-LO (Abramovitz et al., 1993; Coffey et al., 1994).

The novel dimethoxyquinazoline compound WHI-P97 is a rationally designed inhibitor of JAK3 (Sudbeck et al., 1999). WHI-P97 was very well tolerated in mice, and mast cell inhibitory “therapeutic” concentrations of WHI-P97 could be achieved at nontoxic dose levels. Treatment of OVA-sensitized mice with WHI-P97 prevented the development of airway hyper-responsiveness in a dose-dependent fashion. Furthermore, WHI-P97 treatment also resulted in decreased eosinophil recruitment to the airway lumen after the OVA challenge, which is in accordance with our finding that there is poor eosinophil recruitment to the airway lumen of OVA-challenged JAK3-knockout mice. The promising biological activity of WHI-P97 in this mouse model of allergic asthma indicates that further development of WHI-P97 may provide the basis for new and effective treatment as well as prevention programs for allergic asthma in clinical settings.

The biological activity of WHI-P97 in the mouse model of allergic asthma could be explained by the ability of this potent JAK3 inhibitor to block leukotriene synthesis alone, but it is conceivable that inhibition of other JAK3-dependent mast cell responses (Malaviya and Uckun, 1999; Malaviya et al., 1999) contribute to its anti-asthmatic activity as well.

References


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