The Orally Available Spleen Tyrosine Kinase Inhibitor 2-[7-(3,4-Dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-ylamino]-nicotinamide Dihydrochloride (BAY 61-3606) Blocks Antigen-Induced Airway Inflammation in Rodents

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ABSTRACT

Spleen tyrosine kinase (Syk) tyrosine kinase plays essential roles in receptors for Fc portion of immunoglobulins and B cell receptor complex signaling in various inflammatory cells; therefore, inhibitors of Syk kinase may show potential as antiasthmatic/allergic therapeutics. We identified 2-[7-(3,4-dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-ylamino]-nicotinamide dihydrochloride (BAY 61-3606), a potent (IC_{50} = 7.5 nM) and selective inhibitor of Syk kinase. BAY 61-3606 inhibited not only degranulation (IC_{50} values between 5 and 46 nM) but also lipid mediator and cytokine synthesis in mast cells. BAY 61-3606 was highly efficacious in basophils obtained from healthy human subjects (IC_{50} = 10 nM) and seems to be at least as potent in basophils obtained from atopic (high serum IgE) subjects (IC_{50} = 8.1 nM). B cell receptor activation and receptors for Fc portion of IgG signaling in eosinophils and monocytes were also potently suppressed by BAY 61-3606. Oral administration of BAY 61-3606 to rats significantly suppressed antigen-induced passive cutaneous anaphylactic reaction, bronchoconstriction, and bronchial edema at 3 mg/kg. Furthermore, BAY 61-3606 attenuated antigen-induced airway inflammation in rats. Based on these anti-inflammatory effects of BAY 61-3606 both in vitro and in vivo, it was demonstrated that Syk may play a very critical role in the pathogenesis of allergic reactions.

Spleen tyrosine kinase (Syk) is a cytosolic 72-kDa protein tyrosine kinase that plays an essential role in high-affinity IgE receptor (FcεRI)-mediated signaling in mast cells and basophils (Beaven and Baumgartner, 1996). Mast cells developed from Syk-deficient mice conclusively demonstrated the essential role of Syk in FcεRI signaling not only for degranulation but also for lipid mediator synthesis and cytokine production (Costello et al., 1996). Mast cells and basophils produce cytokines important for the late phase allergic reaction (Costello et al., 1996; Shichijo et al., 1999). Mast cell-deficient mice did not exhibit airway inflammation (Kung et al., 1995) or hyperresponsiveness (Kobayashi et al., 2000). These experimental results suggest that mast cells play important roles not only in early but also in late phase allergic reactions and that Syk inhibitors would prevent both phases.

In addition to the critical role of Syk in FcεRI signaling, it has been reported that Syk is essential in signaling from receptors for IgG (FcγRI). Syk-deficient macrophages and neutrophils failed to phagocytose IgG-coated antigen through FcγRI (Crowley et al., 1997; Kiefer et al., 1998). Antigen presentation mediated by antibody and FcεRI (FcγRI and FcεRI) was demonstrated to be around 100-fold more efficient than that in the absence of the antibody (Sallusto and Lanzavecchia, 1994; Maurer et al., 1996). In fact, an antigen/IgE immune complex more efficiently induced airway inflammation than the antigen alone (Zuberi et al., 2000). The bronchoalveolar lavage (BAL) fluid from the ovalbumin (OVA)-challenged mice contained significant amounts of antigen-
specific IgE and IgE-OVA immune complexes. These observations suggest the importance of FcR-mediated phagocytosis/antigen presentation for the deterioration of inflammation and thereby the signaling through Syk to facilitate phagocytosis in maintaining chronic inflammation by repeated and effective antigen presentation.

Furthermore, recent in vivo experiments, in which syk antisense oligodeoxynucleotide treatment inhibited airway inflammation in rats, directly suggest an important role of Syk in pulmonary inflammation (Stenton et al., 2000).

These literature data strongly suggest that Syk is an important enzyme in various inflammation pathways relevant to respiratory diseases and therefore a key target for a novel antiasthmatic therapy. We have recently identified an orally available Syk kinase inhibitor, BAY 61-3606, and in this study, we have characterized the pharmacological profiles of BAY 61-3606 both in vitro and in vivo.

Materials and Methods

Chemicals, Antibodies, and Kits. 2-[7-(3,4-Dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-ylamino]-nicotinamide dihydrochloride (BAY 61-3606) was synthesized by the Department of Chemistry (Bayer Chemicals, Antibodies, and Kits. (44x464) and Lyn (biotin-Ahx-KVEKIGEGTYGV- Fig. 1. Peptide substrates for Syk (biotin-KISDFGLSKALRADE-

61-3606) was synthesized by the Department of Chemistry (Bayer Immunoassay kit, leukotriene (LT) C4/D4/E4, granulocyte-macrophage kit was from Cayman Chemical (Ann Arbor, MI). Anti-human IgG 11032

were plates (105 cells/well) and treated with the indicated concen-

tration of a test compound for 15 min at 37°C. The cells were then

transferred to plates coated with 3 μg/ml human IgG and preblocked with 1% BSA. After incubation for 20 min at 37°C, superoxide production was measured by TopCount by monitoring the luminescence from luminal included in the assay buffer.

U937 Superoxide Production Assay. Assay was implemented as described previously (Yamamoto et al., 2003).

Rats for in Vivo Studies. Male Wistar rats for acute models and male Brown Norway rats for a chronic model (both 6 week old or older) (Charles River Japan, Yokohama, Japan) were used.

Passive Cutaneous Anaphylaxis (PCA) Reactions. Rats were passively sensitized by s.c. injection of 5 ng of anti-DNP IgE in dorsal skin. One day later, a test compound in saline containing 10% cremophor was administered 5 min (i.v.) or 60 min (p.o.) before DNP-BSA administration (1 mg in saline containing 0.5% Evans blue, i.v.). Thirty minutes later, rats were sacrificed, and Evans blue in the sites of sensitization was extracted by formamide overnight at 65°C. Absorbency at 620 nm was measured to determine the amount of Evans blue.
Bronchoconstriction and Bronchial Edema Models in Rats.

Rats were passively sensitized by i.v. injection of 10 μg of SPE-7 1 day before experiments. After urethane anesthesia, main bronchi were exposed and cannulated to measure the change in pulmonary pressure. BAY 61-3606 was administered (p.o.) 60 min before injection of the antigen (1.5 μg of DNP-BSA in saline containing 0.5% Evans blue, i.v.). Change in pulmonary pressure was monitored for 10 min after antigen exposure. Thirty minutes after the challenge, rats were sacrificed and lungs were perfused with 20 ml of phosphate-buffered saline. Evans blue in main bronchi was extracted by formamide and measured as described above.

Airway Inflammation Model in Rats.

Rats were immunized by i.p. injection of OVA in Al(OH)₃ suspension on days 0 and 14. On days 20 and 21, an aerosol of 1% OVA in saline was administered by inhalation. BAL fluid was collected, and cell number and differential counts were determined. BAY 61-3606 was administered (p.o.) from days 0 to 21 (b.i.d.). Dexamethasone was administered (p.o.) from days 0 to 9 and days 18 to 21 (b.i.d.).

Results

Biochemical Characterization. We have identified an orally available Syk kinase inhibitor, BAY 61-3606 (Fig. 1), from a series of imidazopyrimidine analogs. BAY 61-3606 inhibited kinase activity of Syk in a concentration-dependent manner with an IC₅₀ value of 10 nM (Fig. 2a). Lineweaver-Burk analysis confirmed competitive inhibition against ATP (Fig. 2b), and the Kᵥ value was determined as 7.5 nM.

BAY 61-3606 was a highly selective inhibitor of Syk kinase. Other selected tyrosine kinases, Lyn, Fyn, Src, Itk, and Btk, were not inhibited by BAY 61-3606 in concentrations up to 4.7 μM (Table 1).

Inhibition of Cellular Function. In functional assays to measure FcεRI-mediated degranulation in mast cells, BAY 61-3606 inhibited the release of various inflammatory mediators in a concentration-dependent manner. The IC₅₀ values for the FcεRI-mediated hexosaminidase release from a rat basophilic leukemia cell line, RBL-2H3 (Fig. 3a), and serotonin release from rat peritoneal mast cells (Table 2) were found to be 46 and 17 nM, respectively. In RBL-2H3 cells, phosphorylation of Syk was also attenuated (data not shown). None of the reference compounds, dexamethasone (glucocorticoid), disodium cromoglycate (DSCG, mast cell stabilizer), and montelukast (LT antagonist), were found to inhibit hexosaminidase release from RBL-2H3 cells at the concentrations evaluated in this study (Fig. 3a).

In a manner similar to its effect on the degranulation of RBL-2H3 cells and rat peritoneal mast cells, BAY 61-3606 inhibited FcεRI-mediated histamine and tryptase release from HCMCs with IC₅₀ values of 5.1 and 5.5 nM, respectively (Fig. 3, b and c). In addition to the effects on the degranulation, BAY 61-3606 inhibited FcεRI-mediated lipid mediator release (PGD₂ and LTC₄/D₄/E₄) and de novo synthesis of the cytokine GM-CSF in HCMCs (IC₅₀ = 5.8, 3.3, and 200 nM, respectively; Fig. 3, d-f). Montelukast (IC₅₀ = 6.8 μM for histamine and 7.0 μM for tryptase) and DSCG (IC₅₀ = 860 μM for histamine) inhibited degranulation from HCMCs only very weakly, and dexamethasone showed no effect up to 30 μM (Fig. 3, b and c). The potency of reference compounds was weak for lipid mediator synthesis (montelukast, IC₅₀ = 6.8 μM for LT; Fig. 3, d and e). Dexamethasone showed higher potency (IC₅₀ = 6 nM) than BAY 61-3606 for inhibition of cytokine production (Fig. 3f).

BAY 61-3606 was also found to inhibit the degranulation of human freshly isolated basophils. Leukocyte fractions including basophils were isolated from peripheral blood of both high- (>280 U/ml) and low- (<280 U/ml)-serum IgE donors. The expression level of FcεRI in leukocytes from high-serum IgE donors was higher than that from low-serum IgE donors as demonstrated by flow cytometry (data not shown). The leukocytes from both groups responded to anti-IgE stimulation by releasing histamine. BAY 61-3606 inhibited histamine release from leukocytes in high and low IgE groups equipotently, giving IC₅₀ values of 8.1 and 10 nM, respectively (Fig. 4).

BAY 61-3606 was also found to inhibit B cell receptor (BCR)-mediated signaling. The IC₅₀ values for BCR-stimulated increases in intracellular calcium concentration in the

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Kᵢ (nM)</th>
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</thead>
<tbody>
<tr>
<td>Syk</td>
<td>7.5 ± 2.5</td>
</tr>
<tr>
<td>Lyn</td>
<td>5,400</td>
</tr>
<tr>
<td>Fyn</td>
<td>12,500</td>
</tr>
<tr>
<td>Src</td>
<td>6,250</td>
</tr>
<tr>
<td>Itk</td>
<td>4,700</td>
</tr>
<tr>
<td>Btk</td>
<td>5,000</td>
</tr>
</tbody>
</table>

Note: Kᵢ values are shown as mean ± S.E. of two independent experiments.

Table 1: Selectivity profile of BAY 61-3606 in six tyrosine kinase assays. Kᵢ values were shown as mean ± S.E. of two independent experiments.

Fig. 2. Syk kinase assay. The kinase reaction was conducted for 30 min at room temperature in the presence of the indicated concentrations of compounds, 30 μM ATP, and 3 μM biotinylated peptide substrate, which corresponded to the activation loop domain of Syk kinase itself. After termination of the reaction with the addition of the EDTA-containing stop buffer, reaction mixtures were transferred into streptavidin-coated plates to trap biotinylated substrate. After washing, phosphorylation of the substrate was detected by the addition of europium-labeled anti-phosphotyrosine monoclonal antibody (4G10) and the measurement with the multilabel counter ARVO (Wallac Oy, Turku, Finland). a, inhibition curve by BAY 61-3806. b, Lineweaver-Burk plot analysis. Each point indicates mean ± S.D. of two independent experiments.
Ramos human B cell line and for BCR-induced mouse splenic B cell proliferation were 81 and 58 nM, respectively (Table 2). Phosphorylation of Syk in Ramos cells was concentration dependently reduced (data not shown). Dexamethasone showed a similar potent inhibition of B cell growth (IC50 30 nM) as BAY 61-3606, whereas DSCG and montelukast showed no effect up to 10 M (data not shown).

Furthermore, BAY 61-3606 was found to block FcγR-mediated activation of monocytes effectively. BAY 61-3606 inhibited FcγR-mediated superoxide production from a human monocytic cell line, U937 (Fig. 5b) and human monocytes freshly isolated from peripheral blood (Table 2) (IC50 = 52 and 12 nM, respectively). The reference compounds were without effect up to 10 M on the respiratory burst from U937 triggered by FcγRI-aggregation (Fig. 5b). We also examined effects of BAY 61-3606 on the respiratory burst in eosinophils by FcγR stimulation. In mouse, immobilized IgG elicited superoxide production, and it was suppressed by the pretreatment of cells with anti-FcγRIII/II monoclonal antibody 2.4G2 (Fig. 5a). BAY 61-3606 inhibited respiratory burst in a concentration-dependent manner with an IC50 value of 35 nM (Table 2), and it completely suppressed su-

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**Table 2**

Summary of IC50 values of BAY 61-3606 in various cellular assays

<table>
<thead>
<tr>
<th>Cell</th>
<th>Receptor</th>
<th>Readout</th>
<th>IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBL-2H3</td>
<td>FceRI</td>
<td>Hexosaminidase</td>
<td>46 ± 19 (4)</td>
</tr>
<tr>
<td>Rat peritoneal mast cells</td>
<td>FceRI</td>
<td>Serotonin</td>
<td>17 ± 14 (4)</td>
</tr>
<tr>
<td>HCMC</td>
<td>FceRI</td>
<td>Histamine</td>
<td>5.1 ± 1.2 (4)</td>
</tr>
<tr>
<td>HCMC</td>
<td>FceRI</td>
<td>Trypsate</td>
<td>5.5 ± 1.5 (4)</td>
</tr>
<tr>
<td>HCMC</td>
<td>FceRI</td>
<td>PGD2</td>
<td>5.8 ± 1.3 (4)</td>
</tr>
<tr>
<td>HCMC</td>
<td>FceRI</td>
<td>LTs</td>
<td>3.3 ± 0.3 (3)</td>
</tr>
<tr>
<td>Human leukocytes from high IgE donors</td>
<td>FceRI</td>
<td>Histamine</td>
<td>8.1 ± 6.1 (3)</td>
</tr>
<tr>
<td>Human leukocytes from low IgE donors</td>
<td>FceRI</td>
<td>Histamine</td>
<td>10.2 ± 5.2 (7)</td>
</tr>
<tr>
<td>Ramos BCR</td>
<td>BCR</td>
<td>[Ca2+]</td>
<td>81 ± 28 (6)</td>
</tr>
<tr>
<td>Mouse splenic B cells</td>
<td>BCR</td>
<td>Proliferation</td>
<td>58 ± 26 (4)</td>
</tr>
<tr>
<td>Mouse eosinophils</td>
<td>FcγR</td>
<td>Superoxide</td>
<td>35 ± 14 (3)</td>
</tr>
<tr>
<td>U937</td>
<td>FcγRI</td>
<td>Superoxide</td>
<td>52 ± 14 (4)</td>
</tr>
<tr>
<td>Human fresh monocytes</td>
<td>FcγR</td>
<td>Superoxide</td>
<td>12 ± 7 (4)</td>
</tr>
</tbody>
</table>

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Fig. 3. Effects of BAY 61-3606 and reference compounds on the activation of mast cells. a, DNP-BSA-induced degranulation of rat basophilic leukemia cell line, RBL-2H3 cells, which were sensitized with anti-DNA IgE. Hexosaminidase activity in the supernatant was measure by an enzyme assay. Each point indicates mean ± S.E. of four independent experiments. b–f, human cultured mast cells sensitized with human IgE were challenged with anti-human IgE for 30 min (a–d) or 6 h (e). Supernatants were recovered and trypsat activity was measured by an enzyme assay (b). ELISA assays were performed for histamine (a), PGD2 (c), LTC4/D4/E4 (d), and GM-CSF (e). Each point indicates mean ± S.E. of three to four independent experiments.
In Vivo Characterization. PCA assay was carried out to investigate the effect of BAY 61-3606 on mast cell-mediated type-I allergic reactions in rats. Oral administration of BAY 61-3606 dose dependently inhibited the PCA reaction with an ED$_{50}$ value of 8 mg/kg (statistical inhibition at 3 mg/kg; p < 0.05) (Fig. 6a). Although DSCG (30 mg/kg i.v.) showed 60% inhibition of dye leakage by the PCA reaction (Fig. 6b), this compound was not active with oral administration (data not shown). Neither montelukast (10 mg/kg p.o.) nor dexamethasone (0.3 mg/kg p.o.) nor dexamethasone (0.3 mg/kg p.o.) showed any inhibitory activity (Fig. 6, c and d).

The effects of BAY 61-3606 on antigen-induced asthmatic models were investigated. In a bronchoconstriction model, BAY 61-3606 dose dependently inhibited the DNP-BSA-induced increase in pulmonary pressure, and the dose of 3 mg/kg showed statistically significant suppression (Fig. 7). As shown in Fig. 8, BAY 61-3606 also significantly attenuated DNP-BSA-induced bronchial edema at and over dosages of 3 mg/kg. Furthermore, BAY 61-3606 significantly inhibited eosinophil accumulation in the BAL fluid at dose of 30 mg/kg to the same level as dexamethasone at a dose of 0.3 mg/kg (Fig. 9). The inhibitory effect of BAY 61-3606 on the total cell number in BAL fluid was also observed at 30 mg/kg, but it was not statistically significant (data not shown). Body weight of dexamethasone-treated rats decreased gradually during the course of experiment (starting from 140 g, -20 g at the end of the experiment); however, those of BAY 61-3606-treated groups at all dosages were almost same as the vehicle group (~40 to 60 g at the end of the experiment) (data not shown).

Discussion

BAY 61-3606 potently inhibited recombinant Syk kinase activity in an ATP-competitive manner (K$_i$ = 7.5 nM; Fig. 2). Furthermore, more than 626-fold selectivity against several other tyrosine kinases, such as Lyn, Fyn, Src, Itk, and Btk, was demonstrated (Table 1).

The efficacy of BAY 61-3606 on antigen-induced degranulation was confirmed both in RBL-2H3 cells (IC$_{50} = 46$ nM; Fig. 3a) and freshly isolated rat mast cells (IC$_{50} = 17$ nM; Table 2). Also in vivo, BAY 61-3606 was effective in suppressing PCA reactions in skin (Fig. 6). Oral dosing over 3 mg/kg was significantly effective and inhibition was dose-dependent. Furthermore, effects of BAY 61-3606 on lung mast cells were confirmed in two acute asthmatic models; DNP-BSA-induced bronchoconstriction (Fig. 7) and bronchial edema (Fig. 8). Statistic significance was obtained over 3 mg/kg p.o. in both assays. Also in mice, oral administration of BAY 61-3606 suppressed PCA reaction dose dependently (data not shown). This in vivo evidence suggests that BAY 61-3606 may be an effective orally available antiallergy medicine.
Crowley et al., 1997; Kiefer et al., 1998; Lach-Trifilieff et al., 2000) and indicate one aspect of anti-inflammatory profiles of BAY 61-3606.

To confirm an outcome of inhibitory actions of BAY 61-3606 in various types of inflammatory cells, we examined the efficacy of BAY 61-3606 in a rat OVA-induced airway inflammation model. BAY 61-3606 at 30 mg/kg p.o., b.i.d., greatly suppressed accumulation of eosinophils in BAL fluid (Fig. 9). The inhibition was 70% and comparable with that of dexamethasone (0.3 mg/kg p.o., b.i.d.). Thus, not only mast cell-stabilizing activity but also anti-inflammatory activity of BAY 61-3606 was confirmed in vivo. The requirement of higher dose in the chronic model might be related to the pharmacokinetic profile of BAY 61-3606. When rats were treated with a single dose of 10 mg/kg p.o. BAY 61-3606, a maximal concentration of 0.167 mg/l (360 nM) was reached 2 h after administration. Due to relatively fast elimination ($t_{1/2}$ = 1.78 h), we administered the compound twice a day. It should be noted that dexamethasone reduced the increase in body weight of rats, but BAY 61-3606 had no effect during the course of this experiment (data not shown).

The potency of BAY 61-3606 in mast cell and basophil assays was superior to that of DSCG, a widely used mast cell stabilizer as an inhalant (Figs. 3 and 6). Moreover, dexamethasone and montelukast showed little or no effect in many assays selected for this study (Figs. 3, 5, and 6). This difference in efficacy profiles between BAY 61-3606 and other widely used drugs...
make it interesting to try BAY 61-3606 as an alternative anti-asthma/allergic medicine with a novel mechanism of action. In this study, we focused on FcR- and BCR-mediated signals to examine the efficacy of BAY 61-3606. In addition to these signaling cascades, much evidence has been accumulating that Syk also play a critical role in some parts of integrin signaling in neutrophils and macrophages (Vines et al., 2001; Mocsai et al., 2002). Furthermore, an essential role of Syk in IL-1-induced RANTES (regulated on activation normal T cell expressed and secreted) production in human nasal fibroblasts was suggested by an antisense experiment (Yamada et al., 2001). These data increase the value of Syk nasai fibroblasts was suggested by an antisense experiment (Yamada et al., 2001). These data increase the value of Syk.

Fig. 8. Effect of BAY 61-3606 on bronchial edema in rats. Rats were sensitized, treated with BAY 61-3606 and challenged with antigen as described in the legend for Fig. 7. A half-hour after the antigen challenge, rats were sacrificed and lungs were perfused with 20 ml of phosphate-buffered saline. Evans blue in main bronchi was extracted by formamide. Each column indicates the mean and S.E. of five to eight rats. Statistical differences were analyzed using one-way analysis of variance, and differences between groups was assessed using Dunnett’s method (*, p < 0.05; **, p < 0.01). Statistical difference was also analyzed using student’s t test (#, p < 0.05). shown to be essential in collagen-induced activation, which is important for the clotting reaction (Watson et al., 2000). In breast cancer, Syk has been implicated as an important inhibitor of cancer cell growth and metastasis (Coopman et al., 2000). In natural killer cells, expression of dominant-negative Syk attenuated natural cytotoxicity (Bruemmer et al., 1997). In addition, the expression of Syk was reported in several other nonhematopoietic cells (Yanagi et al., 2001). All of these concerns should be clarified in detail in future safety toxicological studies.

In conclusion, BAY 61-3606 is an orally available Syk-selective kinase inhibitor, which exhibits a variety of actions on mast cells, basophils, B cells, eosinophils, and antigen-presenting cells. BAY 61-3606 would have benefits in the treatment of asthma by preventing antigen-induced bronchoconstriction and airway inflammation.

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References


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