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The orally available Syk kinase inhibitor BAY 61-3606 blocks antigen-induced airway inflammation in rodents.

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d) Abbreviations:

BAL, bronchoalveolar lavage; BCR, B cell receptor complex; DSCG, disodium cromoglycate; FcεRI, high affinity type-I receptor for IgE; FcγR, receptors for Fc

portion of IgG; FcR, receptors for Fc portion of immunoglobulins; HCMC, human cultured mast cell; OVA, ovalbumin;

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ABSTRACT

Syk tyrosine kinase plays essential roles in FcR- and BCR-signaling in various inflammatory cells, therefore inhibitors of Syk kinase may show potential as anti-asthmatic/allergic therapeutics. We identified BAY 61-3606, a potent (Ki = 7.5 nM) and selective inhibitor of Syk kinase. BAY 61-3606 inhibited not only degranulation (IC_{50} values between 5-46 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 FcγR 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. Further, 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.

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Spleen tyrosine kinase (Syk) is a cytosolic 72 kDa protein tyrosine kinase, which 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 (FcγR). Syk-deficient macrophages and neutrophils failed to phagocytose IgG-coated antigen through FcγR (Crowley et al., 1997; Kiefer et al., 1998). Antigen-presentation mediated by antibody and FcR (FcγR 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 anti-asthmatic 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*.

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Materials and Methods

Chemicals, antibodies and kits

BAY 61-3606 was synthesized by the Chemistry department of Bayer Yakuhin, Ltd. The structure of BAY 61-3606 (2-[7-(3,4-Dimethoxy phenyl)-imidazo[1,2-c]pyrimidin-5-ylamino]-nicotinamide dihydrochloride) is shown in Fig. 1. Peptide substrates for Syk (biotin-KISDFGLSKALRADENYYKAQTHGKWPVK W) and Lyn (biotin-Ahx-KVEKIGEGTYGVVYK-NH_2), and Boc-Ala-Gly-Pro-Arg-MCA were obtained from Peptide Institute (Osaka, Japan). Europium-labeling kit, $LTC_4/D_4/E_4$ EIA kit, GM-CSF ELISA kit, and Percoll were purchased from Amersham Pharmacia (England, U.K.). Src was purchased from Upstate Biotechnology (Lake Placid, NY). Recombinant human stem cell factor (SCF) and IL-6 were from Peprotech (Rocky Hill, NJ). Histamine ELISA kit was from Immunotech (Marseille, France). PGD2-MOX EIA kit was from Cayman (Ann Arbor, MI). Anti-human IgG $F(ab')_2$ were from ICN (Costa Mesa, CA). All culture mediums and culture supplements were from GIBCO (Scotland, U.K.). Anti-dinitrophenol (DNP) monoclonal mouse IgE (SPE-7), DNP-conjugated bovine serum albumin (DNP-BSA) and other reagents were obtained from Sigma (St. Louis, MO).

Syk kinase assay

Assay was implemented as described before (Yamamoto et al., 2003).

Tyrosine kinase selectivity assays

Fyn, GST-tagged Lyn, and $(Histidine)_6$ (His)-tagged Itk and Btk were expressed in insect cells using the baculovirus-expression system from Pharmingen (San Diego, CA) according to standard protocol. T cell receptor ζ-chain tagged with His (His-zeta) was expressed in E. coli. and purified by Ni^{+}-resin. His-zeta was used as a substrate for all these kinases except Lyn. The concentration of ATP and apparent Km, respectively, were 30, 35 (Lyn), 10, 10 (Fyn), 10, 10 (Src), 40, 36 (Itk) and 30, 29 μM (Btk).

RBL-2H3 degranulation assay

Degranulation assays were performed as previously reported with slight modification (Taylor et al., 1995). Briefly, RBL-2H3 cells were maintained in minimum essential medium supplemented with15% FCS and antibiotics-antimycotics. Cells were seeded in plates and cultured for more than 24 h with 0.3 μg/ml of SPE-7. After washing with PIPES buffer (25 mM PIPES, 125 mM NaCl, 2.7 mM KCl, 5.6 mM glucose, 1 mM $CaCl_2$, 0.1% BSA, pH 7.4), cells were treated with a test compound for 15 min at 37 °C. Cells were then stimulated with 0.1 μg/ml of DNP-BSA for 45 min. The activity of hexosaminidase in the supernatant was measured by an enzyme assay with p-nitrophenyl-ß-D-glucosaminide as a substrate.

Human cultured mast cell (HCMC) assays

HCMC were developed from cord blood stem cells by culturing with 80 ng/ml SCF and 50 ng/ml IL-6 for more than 12 weeks according to the method of Saito (Saito et al., 1995). HCMC were sensitized with 1 μg/ml of human IgE

overnight. After washing cells with Hank's balanced salt solution (HBSS) with 0.1% BSA, HCMC were treated with a test compound for 15 min, and then challenged with 4 μg/ml of anti-human IgE for 30 min (for histamine and PGD_2). For cytokine measurements, cells were resuspended with culture medium and stimulated for 6 h. Supernatants were recovered after centrifugation and kept at -20 °C until ELISA assays for histamine, PGD_2, peptide leukotrienes and GM-CSF. Released tryptase was measured by the enzyme assay with Boc-Ala-Gly-Pro-Arg-MCA as a substrate.

Human basophil degranulation assay

Peripheral blood samples were obtained from atopic and non-atopic volunteers. Leukocytes were purified by dextran sedimentation. Leukocytes were resuspended in Hank's balanced salt solution with 0.1% BSA and seeded into 96 well plates (5×10^5 cells/well). After incubation with a test compound for 15 min, cells were stimulated with 4 μg/ml of anti-human IgE antibody for 30 min at 37 °C. Supernatants were recovered after centrifugation and stored at –20 °C until use. Released histamine was measured by ELISA.

Mouse eosinophil superoxide production assay

Spleens were obtained from IL-5 transgenic mice (kind gift from Dr. Tominaga in Kochi Medical School) (Tominaga et al., 1991). Mouse eosinophils were purified from the splenocytes by negative selection with magnetic beads-labeled with anti-Thy1.2 and anti-B220 monoclonal antibodies by utilizing MACS (Miltenyi). After purification, cells were cultured overnight in RPMI1640

containing 10% FCS. Cells were suspended with HBSS in 96 well plates (10^5 cells/well), and treated with the indicated concentration 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 pre-blocked with 1% BSA. After incubation for 20 min at 37 °C, superoxide production was measured by TopCount by monitoring the luminescence from luminol included in the assay buffer.

U937 superoxide production assay

Assay was implemented as described before (Yamamoto et al., 2002).

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) were used.

Passive cutaneous anaphylaxis (PCA) reactions

Rats were passively sensitized by s.c. injection of 5 ng 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 one 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 PBS (-). Evans blue in main bronchus 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 Ki 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 IC_{50} 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, disodium cromoglycate (DSCG) and montelukast was found to inhibit hexosaminidase release from RBL-2H3 cells at the concentrations evaluated in this study (Fig. 3a).

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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 HCMC with IC_{50} values of 5.1 and 5.5 nM, respectively (Figs. 3, b and c). In addition to the effects on the degranulation, BAY 61-3606 inhibited FcεRI-mediated lipid mediator release (PGD_2 and $LTC_4/D_4/E_4$) and *de novo* synthesis of the cytokine GM-CSF in HCMC (IC_{50} = 5.8 nM, 3.3 nM and 200 nM, respectively, Figs. 3, d-f). Montelukast (IC_{50} = 6.8 μM for histamine and 7.0 μM for tryptase) and DSCG (IC_{50} = 860 μM for histamine) inhibited degranulation from HCMC only very weakly, and dexamethasone showed no effect up to 30 μM (Figs. 3, b and c). The potency of reference compounds was weak for lipid mediator synthesis (Montelukast: IC_{50} = 6.8 μM for LT, Figs. 3, d and e). Dexamethasone showed higher potency (IC_{50} = 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 flowcytometry (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 IC_{50} values of 8.1 and 10 nM, respectively (Fig. 4).

BAY 61-3606 was also found to inhibit BCR-mediated signaling. The IC_{50} values for BCR-stimulated increases in $[Ca^{2+}]_i$ in the 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 (IC_{50} = 30 nM) as BAY 61-3606, while DSCG and montelukast showed no effect up to 10 μM (data not shown).

Further, 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) (IC_{50} = 52 nM and 12 nM, respectively). The reference compounds were without effect up to 10 μM 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 IC_{50} value of 35 nM (Table 2), and it completely suppressed superoxide production at 1 μM, while the efficacy of other reference compounds were none or weak at the concentrations used in this study (Fig. 5a).

In vivo characterization

Passive cutaneous anaphylaxis (PCA) assay was carried out to investigate the effect of BAY 61-3606 on mast cell-mediated type-I allergic reactions in rats.

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Oral administration of BAY 61-3606 dose-dependently inhibited the PCA reaction with an ED_{50} 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.) showed any inhibitory activity (Figs. 6, c and d).

The effects of BAY 61-3606 on antigen (ovalbumin, OVA)-induced asthmatic models were investigated. In a bronchoconstriction model, BAY 61-3606 dose-dependently inhibited the OVA-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 OVA-induced bronchial edema at and over dosages of 3 mg/kg. Further, BAY 61-3606 significantly inhibited eosinophil accumulation in the bronchoalveolar lavage (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 (+40~60 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 (Ki = 7.5 nM, Fig. 2). Further, 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. Further, effects of BAY 61-3606 on lung mast cells were confirmed in two acute asthmatic models; OVA-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 anti-allergy medicine.

Although RBL-2H3 and rat peritoneal mast cells have been used frequently to study the effect of compounds on mast cells, compounds effective on these cells were often less active in human mast cells (Pearce et al., 1982). We thus studied the efficacy of BAY 61-3606 on cord blood stem cell-derived human cultured mast cells (HCMC). HCMC has been recognized to show similar pharmacological characteristics to human lung mast cell (Shichijo et al., 1998), which is one of the primary target cells for asthma therapy. Similarly to rat cells, BAY 61-3606 blocked activation of HCMC by FcεRI-aggregation (Figs. 3, b-f).

The efficacy was not only on degranulation but also on lipid mediators and cytokine production. These results are consistent with the phenotypes observed in Syk deficient mast cells (Costello et al., 1996). Relatively smaller IC50 values in rapid mediator release/synthesis in HCMC compared to those in rat mast cells would indicate heterogeneity of mast cells. Relatively high IC50 value of BAY 61-3606 in GM-CSF production might be due to longer incubation time in this assay or difference in sensitivity to Syk inhibition between readouts. Tryptase has been considered to cause the remodeling in the airway (Sommerhoff, 2001). Reticular basement membrane thickness occurs early in the asthma process even in childhood (Jeffery, 2001). Therefore, effective inhibition of tryptase release by BAY 61-3606 might be effective for the airway remodeling as a long-term efficacy.

We extended the efficacy study to freshly isolated human cells including basophils, which express FcεRI. BAY 61-3606 showed almost similar efficacy on the degranulation of cells from high serum IgE (IC_{50} = 8.1 nM) and low serum IgE (IC_{50} = 10.2 nM) donors (Fig. 4), indicating its efficacy in human including atopic patients. Based on these mast cell and basophil data, BAY 61-3606 is a potent inhibitor of human mast cell/basophil activation by antigen.

BAY 61-3606 also suppressed BCR signaling. The inhibition of BCR engagement-induced calcium mobilization in a human B cell line, Ramos, by BAY 61-3606 (IC_{50} = 81 nM, Table 2) is consistent with the phenotype of Syk-deficient DT40 cells (Takata et al., 1994). Purified splenic B cells responded to anti-IgM antibody to show proliferation. BAY 61-3606 attenuated this cell growth (IC_{50} = 58 nM, Table 2). This is the first pharmacological demonstration, as far

as we know, that inhibition of Syk results in the prevention of a B cell function. These B cell data imply that clonal expansion after BCR-engagement by antigen and further maturation could be attenuated by Syk kinase inhibitors.

BAY 61-3606 concentration-dependently inhibited FcγR-mediated respiratory burst not only in mouse eosinophils (IC_{50} = 35 nM, Table 2) but also in a human monocytic cell line, U937 (IC_{50} = 52 nM, Fig. 5b) and freshly isolated human monocytes (IC_{50} = 12 nM, Table 2). These data are consistent with the previous publications using *syk* antisense or cells derived from KO mice (Matsuda et al., 1996; 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.

In order 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 to 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 BAY 61-3606 (p.o.), 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 show 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). Further, an essential role of Syk in IL-1-induced RANTES production in human nasal fibroblasts was suggested by an antisense experiment (Yamada et al., 2001). These data increase the value of Syk kinase inhibitor as a broad anti-inflammatory agent.

Contrarily to the positive data for drug development described above, evidence exists that indicates risks and potential side effects of Syk kinase inhibitors. Syk deficient mice showed perinatal lethality due to severe systemic hemorrhaging (Cheng et al., 1995; Turner et al., 1995). The mechanism might be the impaired proliferation and migration of endothelial cells (Inatome et al., 2001). In platelets, Syk was 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 (Brumbaugh et al., 1997). In addition, the expression of Syk was reported in several other non-hematopoietic 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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Footnotes

[1]These authors contributed equally to this work.

Legends

Fig. 1. Chemical structure of BAY 61-3606 (2-[7-(3,4-Dimethoxy phenyl)-imidazo[1,2-c]pyrimidin-5-ylamino]-nicotinamide dihydrochloride).

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 mAb (4G10) and the measurement with ARVO. (a) Inhibition curve by BAY 61-3606. (b) Lineweaver-Burk plot analysis. Each point indicates mean ± SD of 2 independent experiments.

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 ± SE of 4 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 (b) tryptase activity was

measured by an enzyme assay. ELISA assays were performed for (a) histamine, (c) PGD_2, (d) $LTC_4/D_4/E_4$, and (e) GM-CSF. Each point indicates mean ± SE of 3 to 4 independent experiments.

Fig. 4. Anti-IgE-induced histamine release from peripheral blood leukocytes. Peripheral blood samples were obtained from high and low IgE volunteers. Leukocytes were purified by the dextran sedimentation. Cells were incubated with a test compound, and then stimulated with anti-human IgE antibody for 30 min. Supernatants were recovered and released histamine was measured by ELISA. Each point indicates mean ± SE of 3 high IgE or 7 low IgE independent donors.

Fig. 5. Effects of BAY 61-3606 and reference compounds on FcγR-mediated superoxide production. (a) Mouse eosinophils were purified from the spleen of IL-5 transgenic mice by negative selection with anti-Thy1.2 and anti-B220 monoclonal antibodies by MACS. After the incubation with inhibitors, cells were transferred to the IgG-coated plate. Superoxide production was monitored by the luminescence from luminol included in the assay buffer. FcB; 10 μg/ml anti-FcgRIII/II (2.4G2), BAY; 0.1 μM BAY 61-3606, Dx; 30 μM dexamethasone, Ds; 10 μM DCSG, Mn; 10 μM montelukast. Each bar indicates mean ± SD of triplicates. A representative experiment was shown. (b) A human monocytic cell line, U937, was treated with IFN-γ and cells were incubated with anti-FcγRI monoclonal antibody on ice. After washing, cells were stimulated with anti-mouse IgG antibody. Superoxide production was detected with the

luminescence as described above. Each point indicates mean ± SE of 4 independent experiments.

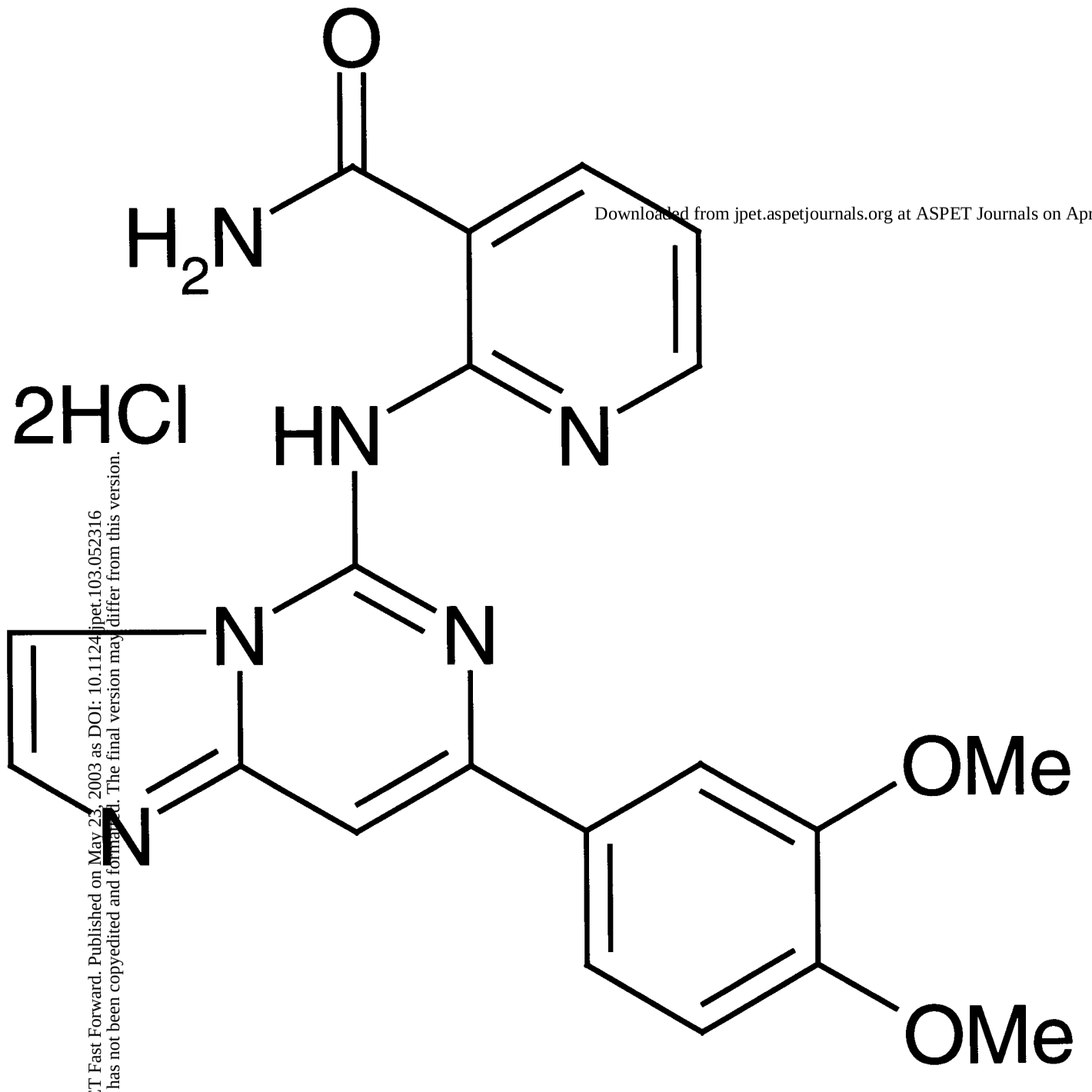
Fig. 6. Effect of BAY 61-3606 and reference compounds on PCA reaction in rats. Rats were passively sensitized by s.c. injection of anti-DNP IgE in the dorsal skin. One day later, a test compound was administered i.v. 5 min or p.o. 60 min before antigen administration (1 mg DNP-BSA and 0.5 % Evans blue in saline). Thirty minutes later rats were sacrificed and Evans blue in skin samples were extracted by formamide. Each column indicates the mean ± SE of 5-8 rats. Statistical differences were analyzed using one-way ANOVA and differences between groups were assessed using Dunnett's method (*: $p<0.05$, **: $p<0.01$).

Fig. 7. Effect of BAY 61-3606 on bronchoconstriction in rats. Rats were passively sensitized by i.v. injection of 10 μg anti-DNP IgE one day before experiment. BAY 61-3606 was administered p.o. 60-70 min before i.v. injection of the antigen (1.5 μg DNP-BSA in saline). Statistical difference at each point was analyzed using Dunnett's test (*: $p<0.05$, **: $p<0.01$). Statistical difference between drug- and vehicle-treated groups was analyzed using two-way ANOVA ($p < 0.001$ in all dose groups).

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 PBS (-). Evans blue in main

bronchi were extracted by formamide. Each column indicates the mean and SE of 5-8 rats. Statistical difference were analyzed using one-way ANOVA 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 (#: $p<0.05$).

Fig. 9. Effect of BAY 61-3606 on OVA-induced airway inflammation in rats. Rats were sensitized by i.p. injection of OVA in aluminum hydroxide gel on days 0 and 14, then inhaled with 1 % OVA in saline on days 20 and 21. BAY 61-3606 was administered p.o. from day 0 to 21 (b.i.d.). Dexamethasone was administered p.o. from day 0 to 9 and day 18 to 21 (b.i.d.). Statistical difference were analyzed using Dunnett's Multiple Comparison Test (*: $p<0.05$, **: $p<0.01$) or Bonferroni's Multiple Comparison Test (#: $p<0.05$).



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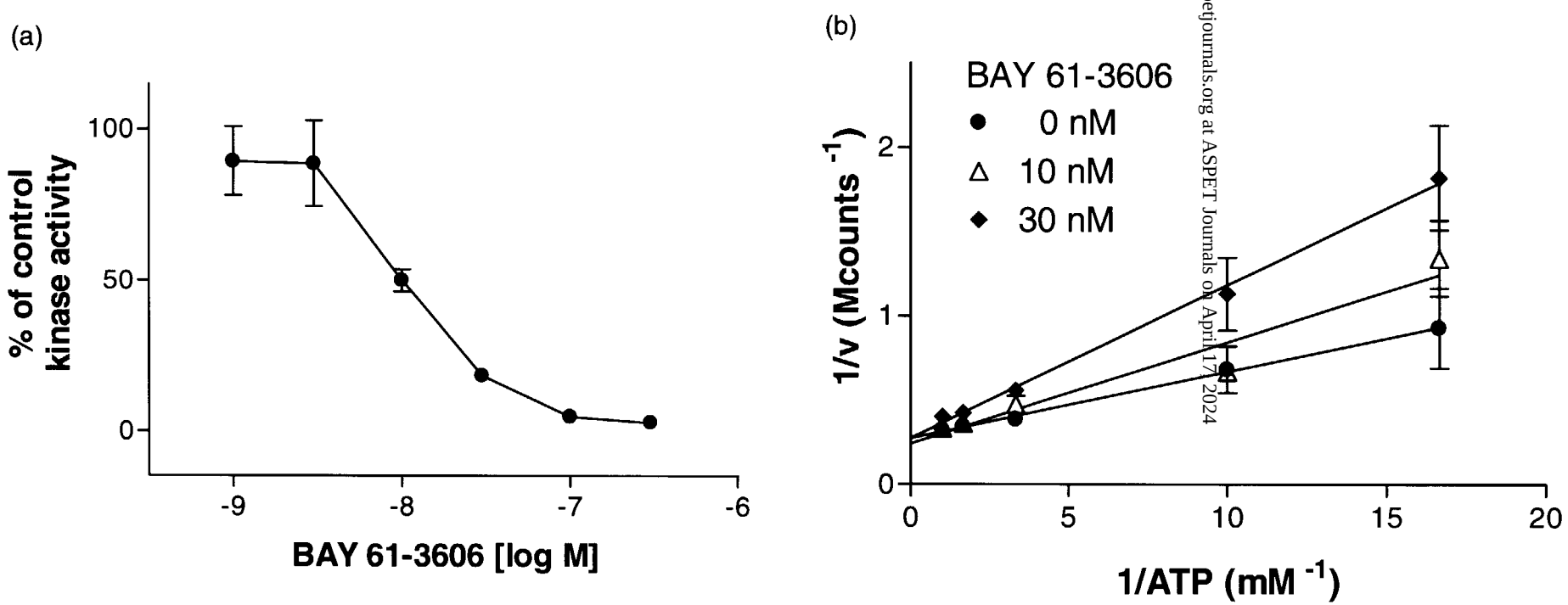

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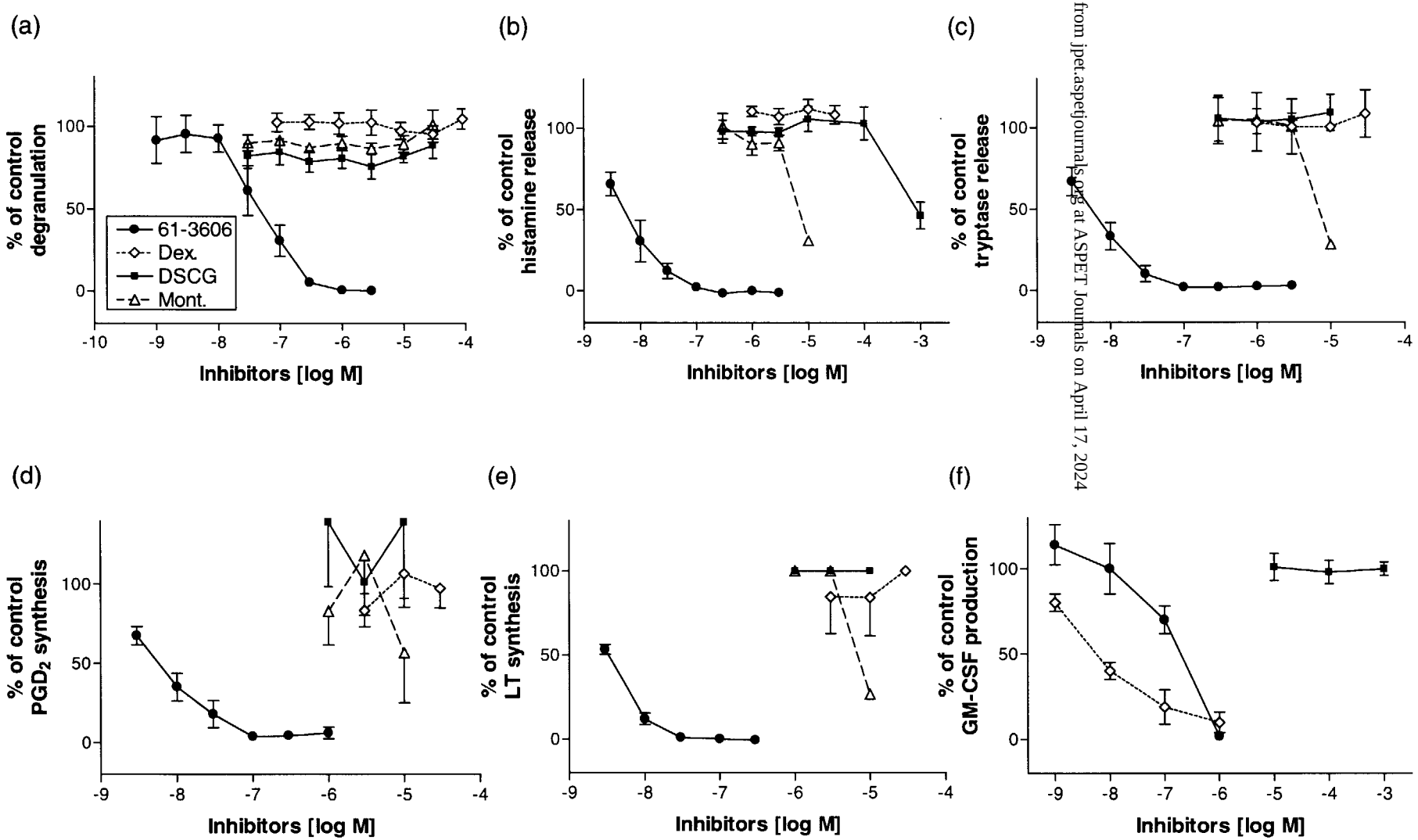

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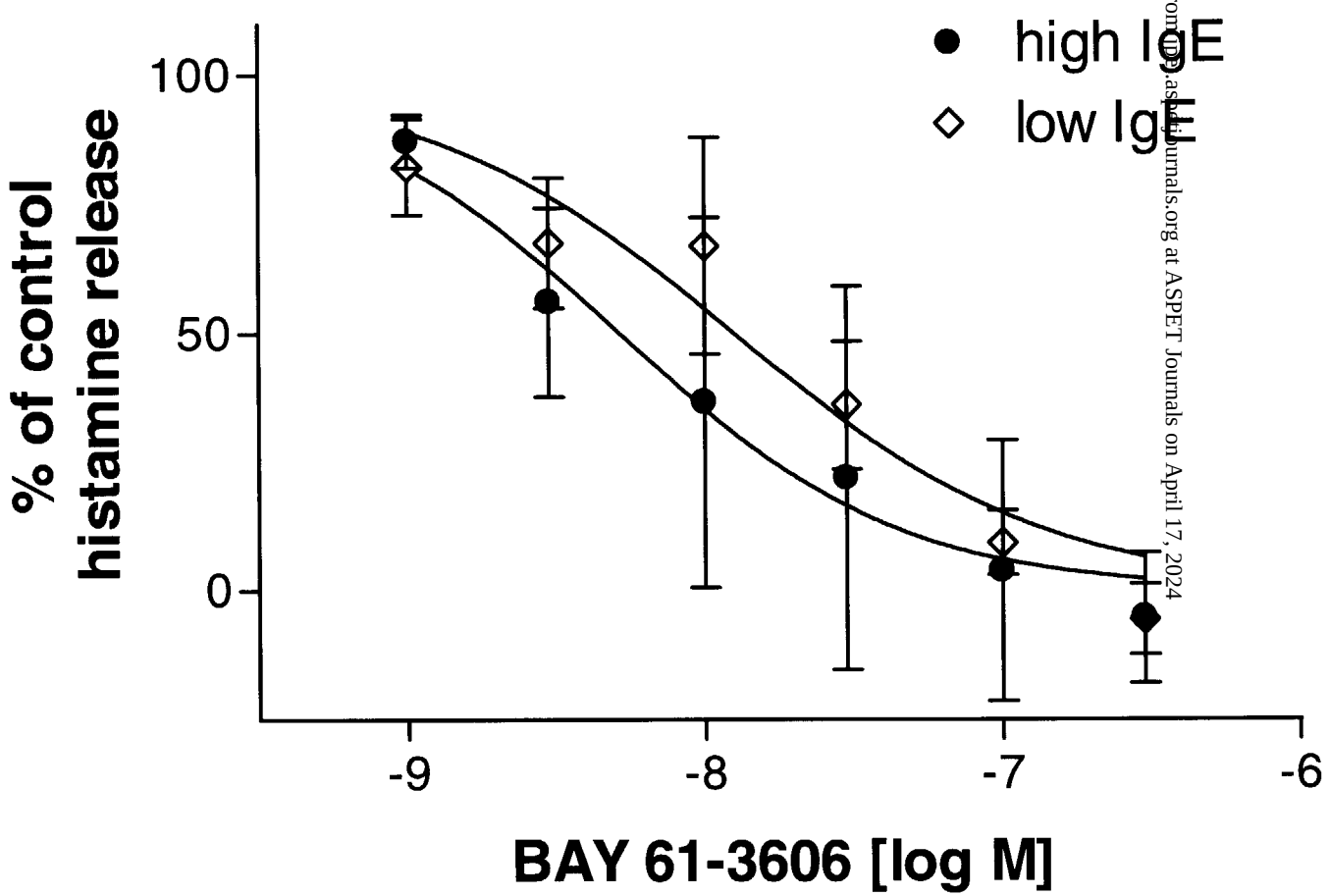

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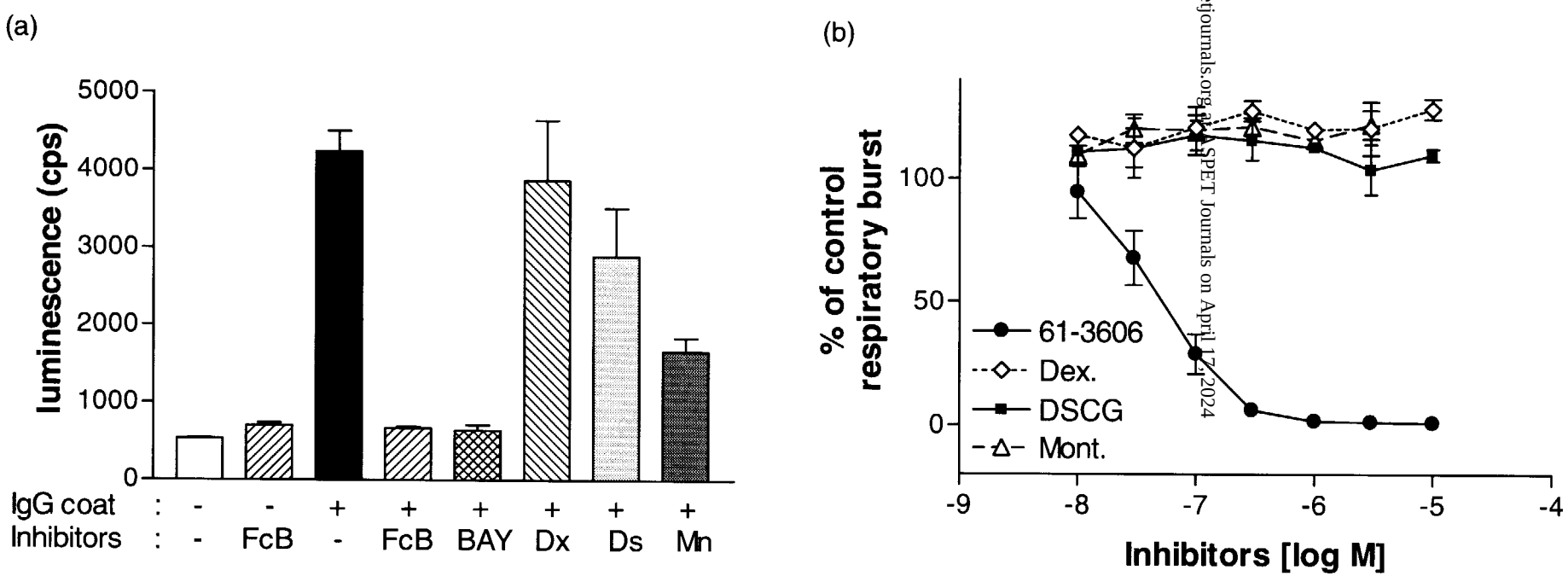

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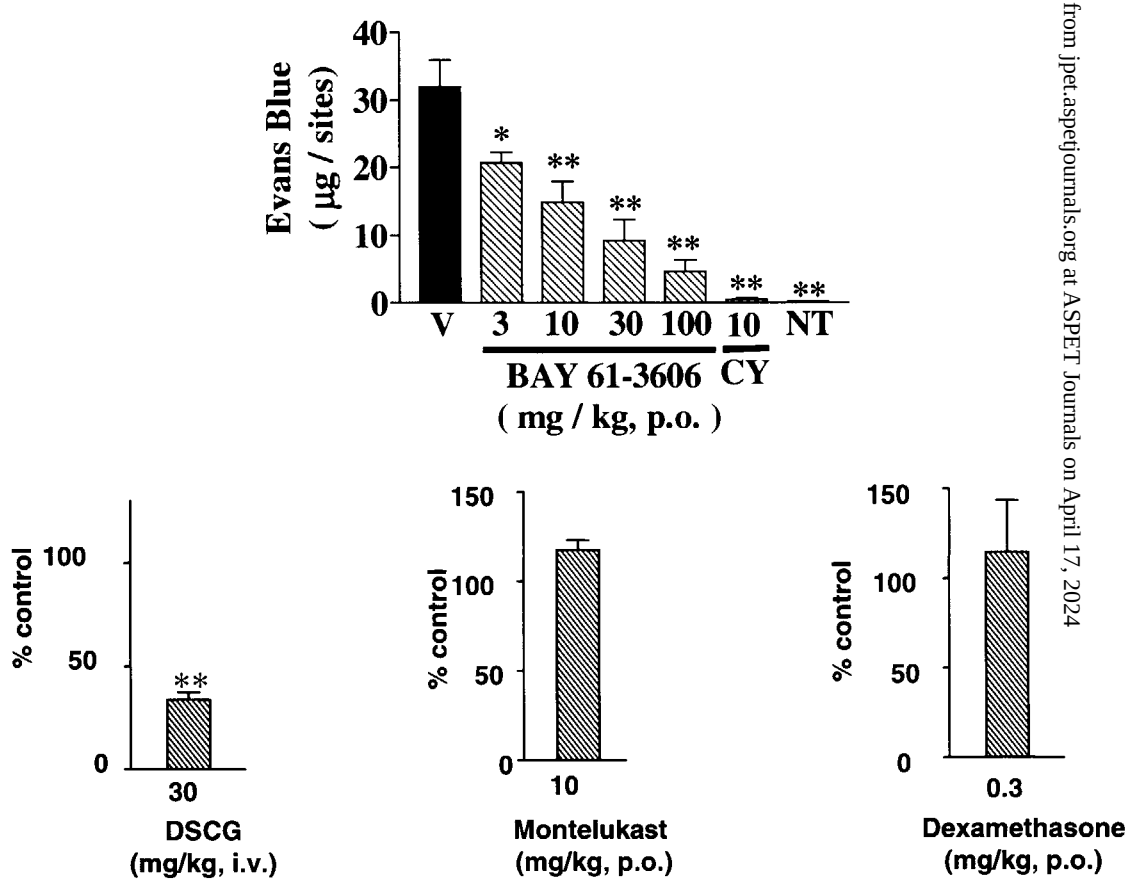

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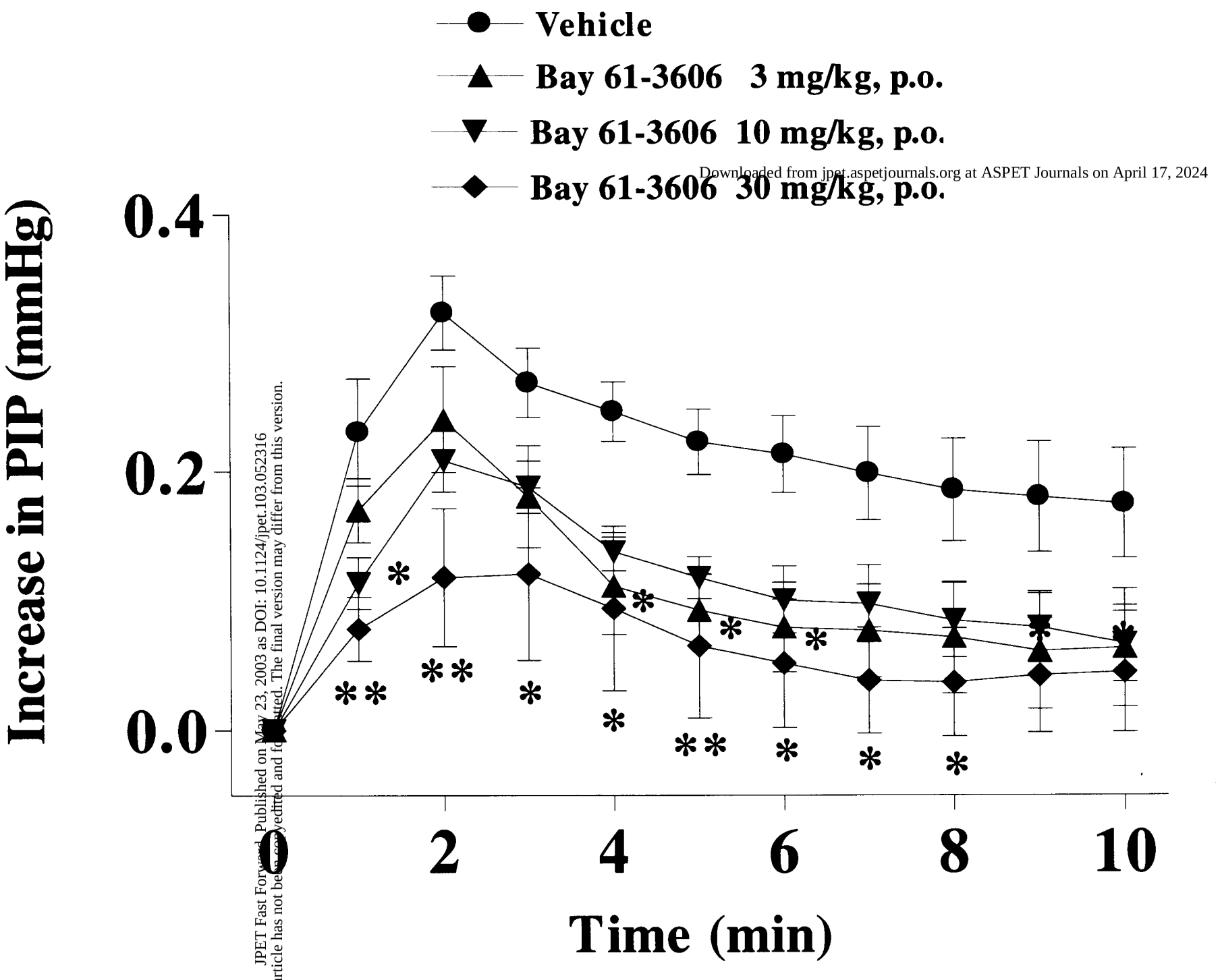

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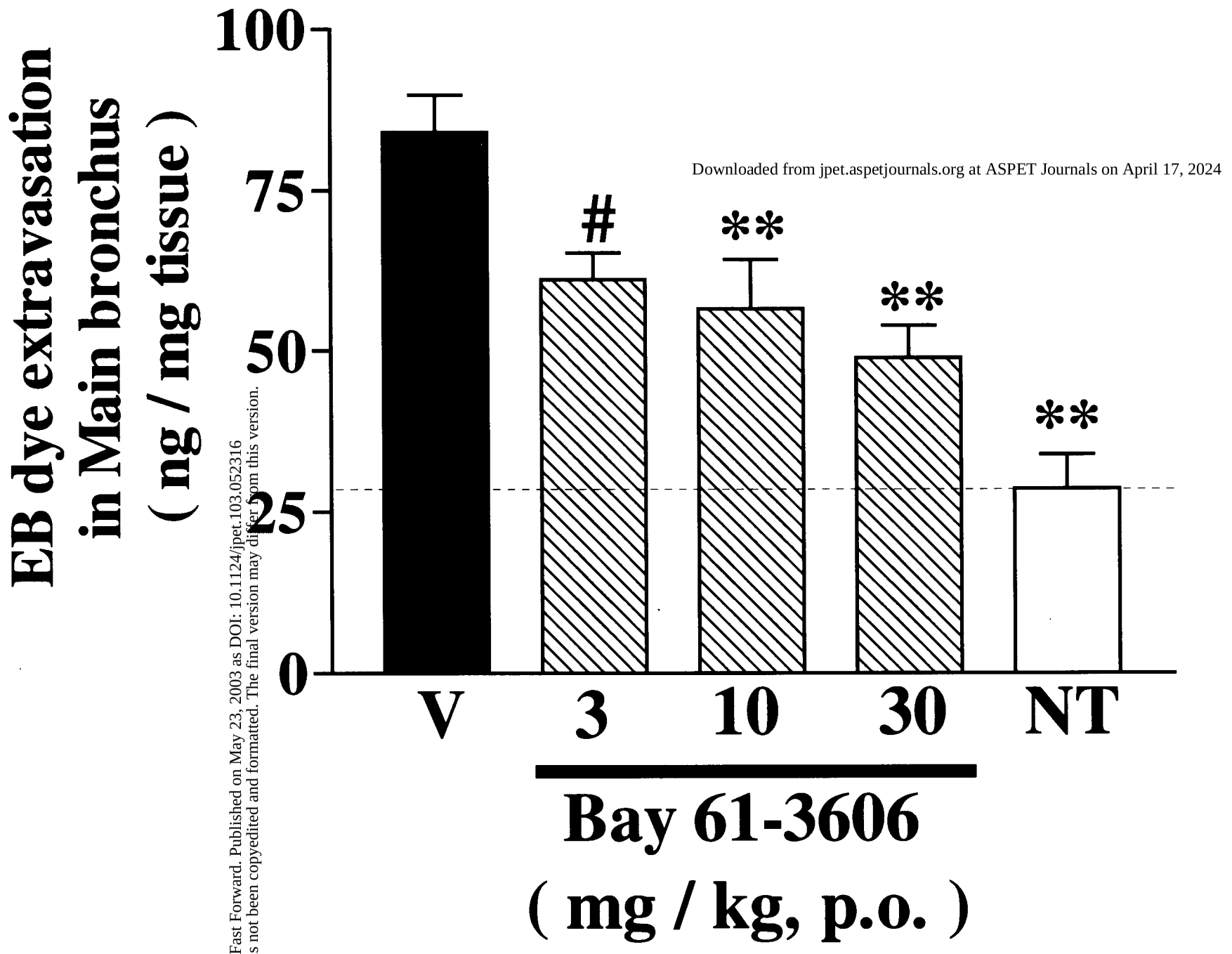

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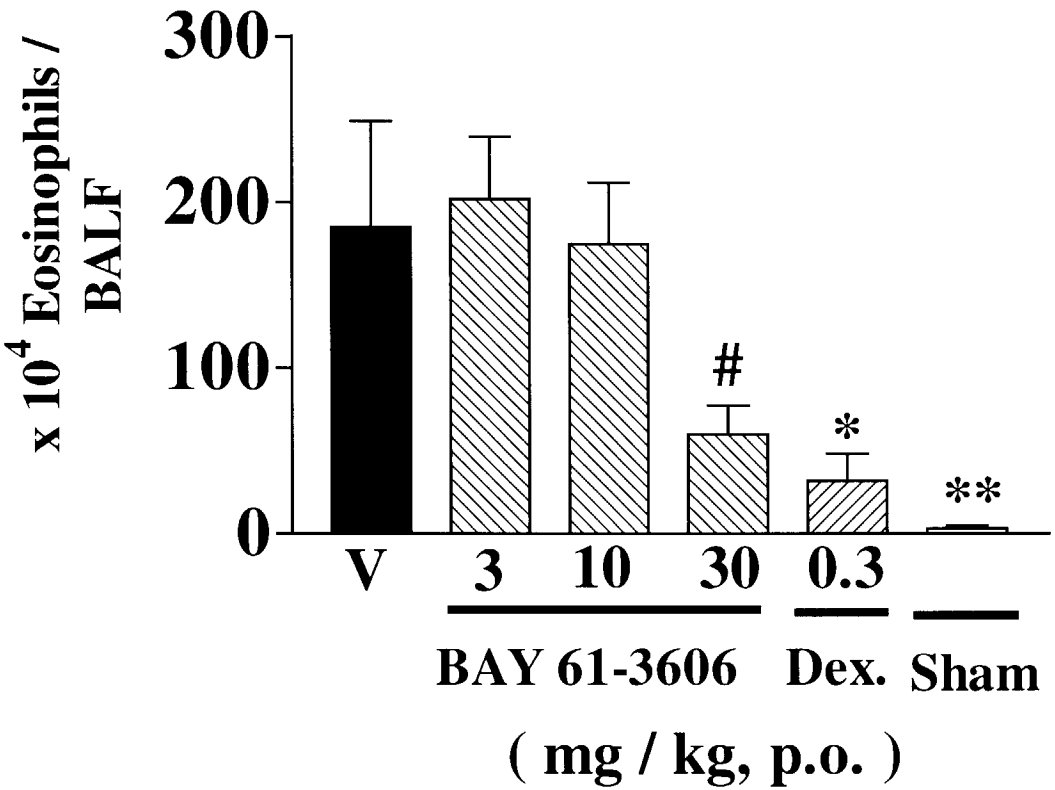

Fig. 9.

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Table 1 Selectivity profile of BAY 61-3606 in 6 tyrosine kinase assays. Ki values were shown as mean ± SE of 2 independent experiments.

Enzymes	Ki (nM)
Syk	7.5 ± 2.5
Lyn	> 5,400
Fyn	> 12,500
Src	> 6,250
Itk	> 4,700
Btk	> 5,000

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Table 2 Summary of IC50 values of BAY 61-3606 in various cellular assays.

Cell	Receptor	Readout	IC_{50} [nM] (n)
RBL-2H3	FcεRI	Hexosaminidase	46 ± 19 (4)
Rat peritoneal mast cells	FcεRI	Serotonin	17 ± 14 (4)
HCMC	FcεRI	Histamine	5.1 ± 1.2 (4)
HCMC	FcεRI	Tryptase	5.5 ± 1.5 (4)
HCMC	FcεRI	PGD_2	5.8 ± 1.3 (4)
HCMC	FcεRI	LTs	3.3 ± 0.3 (3)
HCMC	FcεRI	GM-CSF	200 ± 40 (4)
Human leukocytes from high IgE donors	FcεRI	Histamine	8.1 ± 6.1 (3)
Human leukocytes from low IgE donors	FcεRI	Histamine	10.2 ± 5.2 (7)
Ramos	BCR	$[Ca^{2+}]_i$	81 ± 29 (6)
Mouse splenic B cells	BCR	Proliferation	58 ± 26 (4)
Mouse eosinophils	FcγR	Superoxide	35 ± 14 (3)
U937	FcγRI	Superoxide	52 ± 14 (4)
Human fresh monocytes	FcγR	Superoxide	12 ± 7 (4)