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European Journal of Pharmacology 505 (2004) 223–228

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A novel Syk kinase-selective inhibitor blocks antigen presentation of immune complexes in dendritic cells

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Received 6 September 2004; accepted 12 October 2004
Available online 2 November 2004

Abstract

The initiation of antigen presentation by dendritic cells requires proper internalization of antigens through various mechanisms. Internalization of immune complexes via Fc receptors has been shown to be around 100 times more efficient than the internalization of non-complexed antigens. Spleen tyrosine kinase (Syk) plays an essential role in the signaling cascade initiated by immunoglobulin receptors. We used a selective Syk inhibitor, 7-(3,4-dimethoxyphenyl)-*N*-1*H*-indazol-6-ylimidazo[1,2-*c*]pyrimidin-5-amine dihydrochloride (compound-D), to evaluate the role of Syk in antigen presentation by mouse bone marrow-derived dendritic cells. In line with our expectation, compound-D concentration-dependently inhibited the internalization of immune complexes but not that of antigen itself. Furthermore, when dendritic cells were pretreated with compound-D, the ability of dendritic cells to present immune complex antigens to Th2 cells was attenuated, parallel by a reduced release of interleukin-4 production in Th2 cells. Therefore, Syk kinase activity is a critical component in the process of Fcγ receptor-mediated internalization of immune complex antigens in dendritic cells, and Syk kinase inhibitors may be beneficial in selectively suppressing antibody-mediated antigen presentation in allergic diseases.

Keywords: Syk; Bone marrow-derived dendritic cell, mouse; Immune complex; Fcγ receptor; Antigen internalization; Antigen presentation

1. Introduction

Dendritic cells are the most potent antigen presenting cells and play an important role in allergic diseases (Lambrecht, 2001). Dendritic cells capture antigen via their cell surface receptors such as mannose receptor, integrins, complement receptors and receptors specific for the Fc portion of immunoglobulins (Fc receptors). After internalization, antigens are transported to the lysosome, digested to small peptides, loaded to major histocompatibility complex (MHC) molecules, and presented to T cells as a MHC-peptide complex. It is well known that antigen-specific antibodies enhance antigen presentation efficiency by about 100-fold via Fc receptors by forming antigen–antibody immune complexes (Maurer et al., 1996; Sallusto and Lanzavecchia, 1994).

Spleen tyrosine kinase (Syk) is a cytosolic 72-kDa protein tyrosine kinase that plays an essential role in the high-affinity receptor for immunoglobulin E (IgE), Fcε receptor I, -mediated signaling in mast cells and basophils (Beaven and Baumgartner, 1996; Costello et al., 1996). In addition to its critical role in Fcε receptor I signaling, Syk is essential for the signal transduction initiated by activated receptors for immunoglobulin G (IgG), Fcγ receptors, and involved in the signaling of Fcγ receptor-mediated phagocytosis in macrophages, monocytes, neutrophils, and dendritic cells (Daeron, 1997; Sedlik et al., 2003). During phagocytosis, the interaction of Fcγ receptors with immune complexes induces the local aggregation of Fcγ receptors resulting in the phosphorylation of tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAM) of Fc receptor common γ-subunits. This event leads to the

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doi:10.1016/j.ejphar.2004.10.024

recruitment and activation of Syk, and subsequently downstream signaling molecules are activated (Daeron, 1997; Greenberg et al., 1994). All investigations to define the role of Syk in antigen presenting cells have utilized Syk protein depletion technologies, i.e. Syk-deficient cells derived from knockout mice or antisense treatment. The identification and use of a Syk-selective inhibitor, compound-D, allowed us to expand these studies by focusing on the importance of Syk kinase activity rather than on the entire Syk protein molecule.

2. Materials and methods

2.1. Medium, chemicals, and anti-conalbumin antibody

All cell cultures were maintained in Roswell Park Memorial Institute's medium-1640 (RPMI-1640; Gibco BRL, Scotland, UK) supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, KS), 292 μg/ml L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, St. Louis, MO), hereafter referred to as R10.

7-(3,4-Dimethoxyphenyl)-*N*-1*H*-indazol-6-ylimidazo[1,2-*c*]pyrimidin-5-amine dihydrochloride (compound-D) was synthesized by the Chemistry Department of Bayer Yakuhin (Kyoto, Japan). Supernatant of concanavalin A-stimulated splenocytes (ConA supernatant) was prepared as instructed by American Type Culture Collection (Rockville, MD). Briefly, 2.5×10^6 splenocytes/ml isolated from Wister rats (Japan Charles River, Yokohama, Japan) were cultured in R10 supplemented with 5 μg/ml concanavalin A (Sigma, St. Louis, MO) for 24 h. The culture supernatant was collected, mixed with 20 mg/ml α-methyl-D-mannoside (Sigma), sterilized by filtration, and stored at −30 °C until use. Anti-conalbumin antiserum was produced in conalbumin-immunized rabbits by Sawady Technology (Tokyo, Japan). Rabbit IgG was purified from the antiserum using a Protein A Cartridge (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instruction.

2.2. Animals and cells

All animal experiments were carried out according to the "Guiding principles for the care and use of laboratory animals" formulated by the Japanese Pharmacological Society and the "Guidelines for Animal Experiments" at Bayer Yakuhin. The number of experimental animals was kept to a minimum, and animals were housed in a climate- and light-controlled room with a 12-h light/dark cycle. Six- to eight-week-old male AKR/J mice (Ia^k) were purchased from Seac Yoshitomi (Fukuoka, Japan). Bone marrow-derived dendritic cells were prepared as described (Lutz et al., 1999) with some modifications. Briefly, femurs and tibiae of AKR/J mice were aseptically removed. The bone marrow was flushed out with a syringe. Red blood cells were lysed by a hypotonic shock. Cells were seeded at the density of 2×10^6 cells per 100-mm-dish in 10 ml R10 medium containing 10 ng/ml recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF; PeproTech, Rocky Hill, NJ). At day 3, 10 ml of fresh R10 medium containing 10 ng/ml rmGM-CSF was added to the plates. At days 6 and 8, half of the culture supernatant was collected, washed, and the cell pellet was resuspended in 10 ml of fresh R10 containing 10 ng/ml rmGM-CSF, and placed back onto the original plate. At day 10, dendritic cells were harvested by gentle pipetting. Purity of the obtained dendritic cells was greater than 98% as determined by flow cytometry after staining of cells with anti-CD11c monoclonal antibody (Becton Dickinson, San Jose, CA).

The maintenance of a cloned Th2 cell line, D10.G4.1, restricted to processed conalbumin peptide loaded on Ia^k (D10; purchased from American Type Culture Collection), was performed according to the instructions of the provider. Briefly, D10 cells were pretreated with 5 μg/ml of conalbumin (CA; Sigma), feeder irradiated splenocytes from AKR/J mice in a ratio 2:7, and 10% ConA supernatant. The cells were cultured for 10 days in R10 with 10% ConA supernatant before use.

2.3. Internalization investigation

Fc OxyBURST Green reagent (Molecular Probe, Eugene, OR) and OxyBURST Green H_2HFF BSA (Molecular Probe) were handled according to assay procedures in the product information with small modifications. Dendritic cells were suspended in Hank's balanced solution (pH 7.4; Nissui, Tokyo, Japan) supplemented with 20 mM Hepes (Nacalai Tesque) and 0.1% bovine serum albumin (Sigma). Dendritic cells (3×10^5 cells/well) were pre-incubated with compound-D or the corresponding volume of solvent (0.1% dimethylsulfoxide) for 15 min in 96-well transparent-bottomed black plates (Corning, Corning, NY). The plates with cells were set into FDSS6000 (Hamamatsu Photonics, Hamamatsu, Japan). During measurement of fluorescence intensity (em. 510 nm, ex. 480 nm), Fc OxyBURST Green reagent, or OxyBURST Green H_2HFF BSA was automatically dispensed into each well at 120 or 10 μg/ml, respectively, according to the supplier's recommendation.

2.4. Dendritic cell–T cell co-cultures

Indicated concentrations of conalbumin and anti-conalbumin IgG were co-incubated for 60 min at 37 °C to allow for immune complex formation (conalbumin–immune complex). Dendritic cells (3000 cells/sample) were treated with indicated concentrations of compound-D or 10 μg/ml 2.4G2 (anti-CD16/32 antibody, Fc blocker; Becton Dickinson) for 30 min. The conalbumin–immune complex mixture was added to the dendritic cells and further incubated for 60 min. Finally, D10 Th2 cells (10^5 cells/sample) were added to dendritic cells and immune complex mixture and incubated

for 6 h. As a negative control, dendritic cells were incubated with 0.03 μg/ml conalbumin before the co-culture. Where indicated, dendritic cells were incubated with 10 or 1 μg/ml conalbumin 134–146 peptide (HRGAIEWEGIESG, Custom synthesized by Sawady Technology) before start of the co-culture. Culture supernatant was collected and interleukin-4 was measured by sandwich enzyme-linked immunosorbent assay (ELISA) methods using rat anti-murine interleukin-4 antibody (Becton Dickinson) for coating plates, biotin-conjugated rat anti-murine interleukin-4 antibody (Becton Dickinson), streptavidin-conjugated HRP (Genzyme Techne, Cambridge, MA), and peroxidase detection kit (Sumitomo Bakelite, Tokyo, Japan). Optical density at 450 nm was determined using a microplate reader (Labsystems, Helsinki, Finland).

2.5. Data analysis

Data on the concentration dependence of compound-D on internalization or interleukin-4 production were fitted by nonlinear regression analysis (GraphPad Prism, San Diego, CA) to calculate IC_{50} values.

3. Results

3.1. Immune complex uptake in dendritic cells

We first analyzed Syk function in immune complex (immune complex) internalization of dendritic cells. The Fc OxyBURST Green assay reagent consists of bovine serum albumin covalently linked to dichlorodihydrofluorescein (H_2DCF) and complexed with purified rabbit polyclonal anti-bovine serum albumin IgG antibodies. When these immune complexes are internalized into the phagosome, the nonfluorescent H_2DCF molecules are oxidized to easily detectable green fluorescent dichlorofluorescein (DCF). As shown in Fig. 1A, the fluorescence signal was gradually elevated after the addition of H_2DCF-bovine serum albumin immune complex in the absence of any inhibitor at 1 min. Fluorescence reached its maximum approximately at 21 min and the signal was maintained at least up to 30 min. This increase in fluorescence was diminished by pre-treatment of dendritic cells with the Syk kinase-selective inhibitor, compound-D, in a concentration-dependent manner with an IC_{50} value of 679 nM (Figs. 1A and 2).

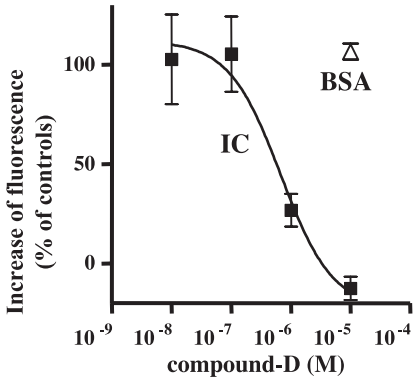

Fig. 2. Compound-D inhibits the internalization of Fc OxyBURST Green reagent (IC). Total increase of fluorescence during the measurement period is indicated as the percentage of control values (absence of compound-D set as 100%, absence of compound-D and antigens as 0%, each point indicates mean±S.E.M. of three independent experiments). Internalization of Oxy-BURST Green H_2HFF BSA (BSA) in the presence of 10 μM compound-D is shown for comparison.

We also tested OxyBURST Green H_2HFF BSA. After addition of H_2HFF-BSA to dendritic cells, fluorescence gradually increased in a similar manner to Fc OxyBURST

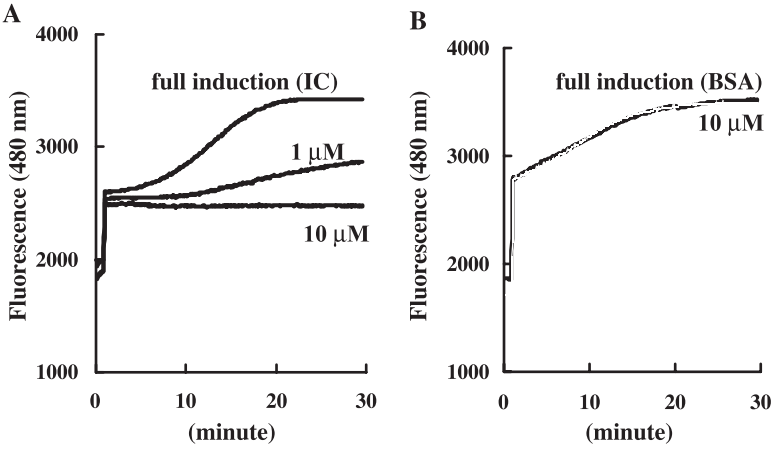

Fig. 1. Inhibition of Fc OxyBURST Green reagent internalization into dendritic cells by compound-D. (A) During measurement of fluorescence intensity, 120 μg/ml Fc OxyBURST Green reagent was automatically dispensed. Internalization was detected fluorometrically (480 nm). The concentrations of compound-D are indicated in the graph and "full induction (IC)" indicates the absence of inhibitor. (One representative data set from three analogous experiments with similar results is shown). (B) During measurement of fluorescence intensity, 10 μg/ml OxyBURST Green H_2HFF BSA was automatically dispensed. The internalization was detected fluorometrically (480 nm). "Full induction (BSA)" (black line) indicates the absence of inhibitor and "10 μM" (gray line) indicates 10 μM of compound-D. (One representative data set from three analogous experiments with similar results is shown).

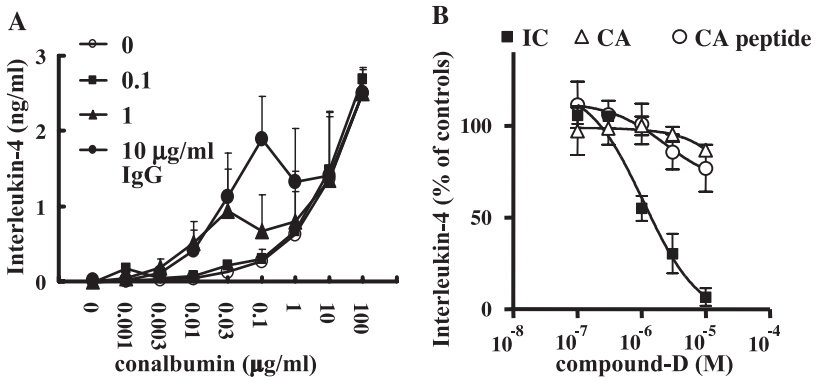

Fig. 3. Compound-D inhibits antigen presentation of immune complex by dendritic cells to T cells. (A) Various concentrations of conalbumin and anti-conalbumin IgG were mixed for 1 h. Dendritic cells were plated in the presence of the mixture for 30 min before D10 T cells were added. Six hours later, culture supernatants were collected and interleukin-4 was measured by ELISA (each point indicates mean+S.E.M. of three independent experiments). (B) Compound-D inhibits antigen presentation of immune complex but not of conalbumin or conalbumin peptide. Immune complex was formed by incubating 0.03 μg/ml conalbumin with 1 μg/ml anti-conalbumin IgG. Dendritic cells were left untreated or treated with compound-D for 30 min. Next, dendritic cells were incubated with conalbumin-immune complex (IC), 10 μg/ml conalbumin (CA), or 1 μg/ml conalbumin peptide (CA peptide) for 60 min. D10 Th2 cells were added and 6 h later culture supernatant was collected and interleukin-4 was measured by ELISA. Production of interleukin-4 is indicated as the percentage of control values (absence of compound-D set as 100%, absence of compound-D and conalbumin-immune complex but 0.03 μg/ml conalbumin as 0%, each point indicates mean±S.E.M. of three independent experiments).

Green assay reagent, however this increase was not influenced by pre-treatment of cells with 10 μM compound-D (Figs. 1B and 2).

3.2. Immune complex formation

In order to establish a system to investigate the function of Syk in antigen presentation of immune complexes in dendritic cells, we first incubated various concentrations of conalbumin and anti-conalbumin rabbit IgG in order to allow conalbumin–immune complex formation. Dendritic cells were incubated with this mixture and co-cultured with D10 cells. Six hours later, the culture supernatant was collected and interleukin-4 was measured (Fig. 3A). Dendritic cells incubated with conalbumin in the absence of antibody activated T cells to secrete interleukin-4 in a conalbumin concentration-dependent manner. When conalbumin complexed to anti-conalbumin IgG was used, interleukin-4 production was even further enhanced, and IL-4 production was most pronounced at a conalbumin/IgG ratio of approximately 1: 100 (Fig. 3A).

We next investigated the effect of compound-D on conalbumin–immune complex endocytosis and ensuing presentation to T cells. We chose an experimental condition of 0.03 μg/ml conalbumin plus 10 μg/ml anti-conalbumin IgG, in order to induce significant interleukin-4 production, and in order to keep the cytokine production near the basal induced by the corresponding concentration of conalbumin alone. First, we pre-incubated dendritic cells with or without compound-D. Dendritic cells were then incubated with conalbumin–immune complex and co-cultured with D10 cells. As shown in Fig. 3B, antigen presentation induced by conalbumin–immune complex was inhibited by compound-D in a concentration-dependent manner with an IC_{50} value of 1.08 μM. In order to determine whether compound-D directly suppressed interleukin-4 release from T cells or whether compound-D blocked non-IgG-mediated internalization, we cultured dendritic cells pretreated with compound-D in the presence of a high concentration of conalbumin (10 μg/ml) or conalbumin peptide, both of which inducing comparable interleukin-4 production as immune complex, and co-cultured them with D10 cells. As shown in Fig. 3B, the effect of compound-D on conalbumin- and conalbumin-peptide-induced interleukin-4 production was minimal and did not reach 50% inhibition at 10 μM.

4. Discussions

We previously described that a Syk selective inhibitor, BAY 61-3606, attenuates Fc receptor- and B cell receptor-mediated activation of mast cells, basophils, monocytes, eosinophils and B cells (Yamamoto et al., 2003). An analog of BAY 61-3606, compound-D, possessed comparable potency and selectivity as BAY 61-3606 (Table 1). In the

Table 1
Ki values of compound-D and BAY 61-3606 in various tyrosine kinase assays

Ki values (nM)	Syk	Lyn	Fyn	Src	Itk	Btk	PKCα	PKCθ
Compound-D	10	750	2050	5000	>9500	>10,000	>15,000	>15,000
BAY 61-3606	7.5	>5400	>12,500	>6250	>4700	>5000	90	>15,000

Inhibition constants (Ki) of compound-D and BAY 61-3606 for various kinases (data are mean values of at least three independent determinations).

present study, we aimed at investigating the efficacy of this new Syk kinase inhibitor on Fcγ receptor-mediated antigen internalization in mouse dendritic cells and ensuing antigen presentation to Th2 type T cells.

Fc OxyBURST Green reagent allows the measurement of the kinetics of Fc receptor-mediated internalization and the subsequent oxidative burst directly in the phagovacuole. When this immune complex binds to Fc receptors, the nonfluorescent H_2DCF molecules are internalized into the phagovacuole and subsequently oxidized to the green fluorescent dye DCF. Compound-D clearly blocked this fluorescence elevation in a concentration-dependent manner.

In order to exclude the possibility that compound-D blocked the oxidization step of fluorescence dye nonspecifically and that compound-D was directly toxic to dendritic cells, we also tested OxyBURST Green H_2HFF BSA, that consists of bovine serum albumin coupled to H_2HFF, in the same assay system. Fluorescence of H_2HFF-bovine serum albumin also increased after addition to dendritic cells following a similar time-course to that of Fc OxyBURST Green reagent, but there was no attenuation of the signal in the presence of 10 μM compound-D (Figs. 1B and 2). This result demonstrated that compound-D selectively inhibited Fc receptor-mediated increases in fluorescence without inducing cytotoxicity, and more importantly, that Syk might not play an important role in non-Fc receptor-mediated internalization of antigen. We also tested compound-D and its close analogues with regard to nonspecific cytotoxicity on two types of cells, HeLa cell line and mouse eosinophils. Toxicity was not observed up to 10 μM (data not shown).

Previously, we reported that the inhibition of Syk kinase activity resulted in the suppression of allergic and asthmatic reactions in vivo (Yamamoto et al., 2003). There may be many mechanisms by which a Syk kinase inhibitor might exert such in vivo efficacy. In this study, we focused on antigen presentation by dendritic cells to activate Th2 cells, which play a central role in allergic diseases. Interleukin-4 was measured as a representative cytokine from activated Th2 cells. When conalbumin and anti-conalbumin IgG were mixed and exposed to dendritic cells, the ratio of conalbumin and IgG, which resulted in peaks of interleukin-4 liberation from D10 cells, was approximately 1:100 (Fig. 3A). This implies that abundant production of interleukin-4 was due to immune complex formation and more efficient internalization of immune complex might have occurred in the presence of immunocomplex as opposed to that seen with conalbumin alone. Compound-D inhibited the interleukin-4 production from D10 Th2 cells when dendritic cells were loaded with conalbumin–immune complex but not when they were loaded with a high concentration of conalbumin alone or a conalbumin epitope peptide (Fig. 3B). These results demonstrate that compound-D is neither toxic to D10 nor to dendritic cells and indicate that compound-D selectively suppresses FcR-mediated internalization of immune complex. In order to confirm that conalbumin–immune complex internalization in dendritic cells was indeed mediated by Fcγ receptors, dendritic cells were treated with conalbumin–immune complex in the presence of anti-Fcγ receptor II and III monoclonal antibody (clone 2.4G2) followed by co-culture with D10. This antibody suppressed interleukin-4 production from D10 (data not shown). These results imply that the target cells of compound-D are dendritic cells rather than T cells and that the antigen presentation of immune complex is at least partially mediated by Fcγ receptors II and/or III and depends on Syk.

It was reported that human T cells from zeta-associated protein-70 (ZAP-70)-deficient patients expressed high levels of Syk, but no detectable Syk activity was found in healthy controls (Noraz et al., 2000). We confirmed that Syk expression in D10 was below the detection limit in normal, CD3/CD28-stimulated, and phorbol-myristate acetate-stimulated conditions, although normal splenocytes from AKR/J mice showed abundant expression when examined by Western blotting (data not shown). When D10 were stimulated with plate-coated anti-CD3 antibodies, the effect of compound-D on IL-4 production was fairly weak with an IC_{50} value of more than 10 μM (data not shown). Therefore, it is unlikely that compound-D suppresses ZAP-70, which is the closest tyrosine kinase homologue to Syk and is important in T cell receptor signaling.

In conclusion, our data are in line with a dominant role of Syk kinase activity in Fcγ receptor-mediated endocytosis in dendritic cells, and we pharmacologically confirmed the importance of Syk kinase activity, rather than the existence of Syk molecule itself as shown in knock out studies (Sedlik et al., 2003). This study further indicates a potential benefit for Syk kinase inhibitors in the treatment of allergic diseases.

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