A novel druglike spleen tyrosine kinase binder prevents anaphylactic shock when administered orally

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Background: The spleen tyrosine kinase (Syk) is recognized as a potential pharmaceutical target for the treatment of type I hypersensitivity reactions including allergic rhinitis, urticaria, asthma, and anaphylaxis because of its critical position upstream of immunoreceptor signaling complexes that regulate inflammatory responses in leukocytes.
Objective: Our aim was to improve the selectivity of anti-Syk therapies by impeding the interaction of Syk with its cellular partners, instead of targeting its catalytic site.
Methods: We have previously studied the inhibitory effects of the anti-Syk intracellular antibody G4G11 on FcεRI-induced release of allergic mediators. A compound collection was screened by using an antibody displacement assay to identify functional mimics of G4G11 that act as potential inhibitors of the allergic response. The effects of the selected druglike compounds on mast cell activation were evaluated *in vitro* and *in vivo*.
Results: We discovered compound 13, a small molecule that inhibits FcεRI-induced mast cell degranulation *in vitro* and anaphylactic shock *in vivo*. Importantly, compound 13 was efficient when administered orally to mice. Structural analysis, docking, and site-directed mutagenesis allowed us to identify the binding cavity of this compound, located at the interface between the 2 Src homology 2 domains and the interdomain A of Syk.
Conclusion: We have isolated a new class of druglike compounds that modulate the interaction of Syk with some of its macromolecular substrates implicated in the degranulation pathway in mast cells. (J Allergy Clin Immunol 2008;122: 188-94.)

Key words: *Allergy and inflammation, anaphylactic shock, degranulation, druglike compound, mast cells/basophils, tyrosine kinase Syk*

The protein tyrosine kinase (PTK) spleen tyrosine kinase (Syk) is a cytoplasmic protein that is a key mediator of immunoreceptor signaling in cells involved in inflammation such as B cells, mast cells, macrophages, and neutrophils. In mast cells and basophils, cross-linking of FcεRI with IgE and antigen induces phosphorylation of FcεRI immunoreceptor tyrosine-based activation motifs (ITAMs) that form a binding site for Syk, which is subsequently activated. Active Syk then phosphorylates many substrates, including the adapter proteins linker for activation of T cells (LAT), Src homology 2 domain–containing leukocyte protein of 76 kd (SLP-76), and Vav, leading to the activation of several signaling pathways, such as those of phospholipase Cγ (PLC-γ), phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated kinase (Erk), c-jun N-terminal kinase (JNK), and p38. These pathways lead eventually to degranulation; the synthesis and release of lipid mediators; and the production and secretion of cytokines, chemokines, and growth factors by mast cells and basophils.[1,2] Syk was demonstrated to regulate FcεRI signaling positively,[3] suggesting that it could be an excellent target for treating allergic disorders. Pharmacologic inhibitors of Syk kinase activity bearing therapeutic potential have been developed.[4] However, because Syk is widely distributed in different cell types, the risk must be reduced of unwanted consequences of inhibiting the catalytic activity of this kinase on various physiologic functions such as cell differentiation, adhesion, and proliferation.[5,6] To this end, new approaches must be developed for the discovery of a novel class of safer yet effective Syk inhibitors.

In a previous study,[7] we reported the inhibitory effects of the intracellular antibody (intrabody) G4G11 on the FcεRI-induced release of allergic mediators in mast cells. The single-chain variable domain (scFv) G4G11 was isolated from a combinatorial library screened against a recombinant protein containing Syk Src homology 2 (SH2) domains.[8] We hypothesized that G4G11 binds to a region of Syk that interacts with partners essential for the degranulation pathway. Considering the limitations to the use of intrabodies in therapy, such as the efficient transfer of the antibody-encoding gene into target cells,[9] we wished to isolate druglike compounds that act as functional mimics of G4G11. We identified compound 13 (C-13), a small molecule able to interfere *in vitro* with the interaction of G4G11 with Syk. We predicted, *in silico*, the likely binding site of C-13 on Syk, guided by the localization of G4G11 epitope, and we further validated these theoretical predictions via site-directed mutagenesis. Our results show that C-13 displays strong inhibitory effects on IgE-mediated mast cell degranulation and is also able to interfere

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Abbreviations used
BMMC: Bone marrow–derived mast cell
C-13: Compound 13
ITAM: Immunoreceptor tyrosine-based activation motif
LAT: Linker for activation of T cells
PCA: Passive cutaneous anaphylaxis
PI3K: Phosphatidylinositol 3-kinase
PLC-γ: Phospholipase Cγ
PSA: Passive systemic anaphylaxis
PTK: Protein tyrosine kinase
scFv: Single-chain variable domain
SH2: Src homology 2
SLP-76: Src homology 2 domain–containing leukocyte protein of 76 kd
Syk: Spleen tyrosine kinase

in vivo with passive cutaneous and passive systemic anaphylaxis (PSA) in BALB/c mice.

METHODS

Chemicals and antibodies

A chemical library of 3000 molecules (a diverse subset) was purchased from ChemBridge, Inc (San Diego, Calif). Stock solutions of small molecules were prepared at 10 mmol/L in dimethyl sulfoxide, except for C-13 (methyl 2-{5-[(3-benzyl-4-oxo-2-thioxo-1,3-thiazolidin-5-ylidene)methyl]-2-furyl}benzoate; ChemBridge ID number 6197026) and the irrelevant chemical (ChemBridge ID number 5522980), which were prepared in dimethylformamide. All reagents unless otherwise mentioned were from Sigma (St Louis, Mo). The hapten dinitrophenyl was purchased from Calbiochem (Merck, Darmstadt, Germany). Antibody reagents are described in the Methods section in this article's Online Repository at www.jacionline.org.

ELISA-based antibody displacement high-throughput assay (WO 2005106481)

Recombinant glutathione S-transferase:Syk 6-242 fusion protein[8] was immobilized on an ELISA plate at a final concentration of 10 μg/mL. For the screening of the chemical library, small molecules diluted in PBS at a final concentration of 10 μmol/L were added to the wells for 1 hour at room temperature before adding myc-tagged scFv G4G11 at final concentration of 100 nmol/L for 1 additional hour. The binding of G4G11 to Syk was evaluated by adding horseradish peroxidase–conjugated mAb 9E10, which detects the amino acid sequence EQKLISEEDLN of human c-myc protein located at the C-terminal end of the scFv. To generate Syk mutants, site-directed mutagenesis was performed on the glutathione S-transferase:Syk 6-242 protein, and the binding of G4G11 to the mutants was evaluated in the presence of 5 μmol/L C-13.

Cells, culture conditions, and functional assays

The mouse IgE antidinitrophenyl mAb 2682-I was used as a hybridoma culture supernatant, which contained 1 μg/mL IgE. Femoral bone marrow cells were collected and cultured in Opti-MEM medium (Gibco-Invitrogen Corp, Carlsbad, Calif) supplemented with 10% FBS and 4% supernatant of X63 transfectants secreting murine IL-3. RBL-2H3 rat basophilic leukemia cells (American Type Culture Collection) were maintained as monolayer cultures in RPMI-1640 medium supplemented with 10% FBS (Gibco). Measurements of β-hexosaminidase release in RBL-2H3 cells were performed as described,[7] except that after 12 to 16 hours of incubation with antidinitrophenyl IgE (0.5 μg/mL), cells were incubated for 90 minutes at 37°C in RPMI medium supplemented with the indicated concentrations of C-13 or DMF (0.25%). Cells were challenged for 45 minutes with dinitrophenyl-BSA (50 ng/mL) or ionomycin (1.5 μmol/L). Bone marrow–derived mast cells (BMMCs) were incubated for 1 hour at 37°C with antidinitrophenyl IgE (100 ng/mL). They were then incubated with C-13 (3 μmol/L) or DMF (0.3%) for 3 hours at 37°C and challenged with various concentrations of dinitrophenyl-BSA. β-Hexosaminidase release was measured 10 minutes later, and TNF-α was titrated by cytotoxicity assay on L929 cells as described,[10] 3 hours after challenge.

Induction of anaphylaxis

Female BALB/c mice (6-8 weeks old) were purchased from Charles River (L'Arbresle, France) and kept at the Institut de Recherche en Cancérologie de Montpellier animal house under pathogen-free conditions. Protocols for IgE-dependent PSA and passive cutaneous anaphylaxis (PCA) were performed as described.[11] Briefly, mice were injected intravenously with 100 μg IgE (SPE-7; Sigma) in 200 μL PBS for PSA, or intradermally with 25 ng IgE in 10 μL PBS for PCA, and challenged 24 hours later with intravenous injection of 1 mg dinitrophenyl–keyhole limpet hemocyanin in 2% Evans blue. C-13, irrelevant chemical, or vehicle was administered either orally (PSA) in 200 μL carboxymethylcellulose 1% or locally (PCA) to the ear skin in acetone/olive oil (4:1) 1 hour before the challenge. Animals were killed 20 minutes after the challenge. Ears were removed and minced, and Evans blue was extracted by overnight incubation in formamide at 80°C. For temperature measurements in PSA, C-13 (100 mg/kg) or vehicle was administered orally 3 hours before the challenge performed in the absence of Evans blue. Temperature was monitored by using an electronic thermometer with a rectal probe (YSI, Yellow Springs, Ohio) before challenge, and for 60 minutes afterward, before sacrifice. Absorbance was measured at 610 nm.

Structural studies

Druggable pockets were predicted with Q-SiteFinder (University of Leeds, Leeds, United Kingdom)[12] and ICM (Molsoft LLC, La Jolla, Calif).[13] C-13 was docked by using LigandFit (Accelrys Software Inc, San Diego, Calif)[14] and Surflex (Biopharmics LLC, San Francisco, Calif).[15] The top 20 poses were analyzed and 1 consensus pose is presented in Fig 1, *A*. Images were generated with PyMol (DeLano Scientific LLC, Palo Alto, Calif).

Statistical analysis

Averaged numeric data are expressed as means ± SDs. The Student *t* test was used to determine the statistical significance of differences between groups.

Immunoprecipitations, *in vitro* kinase assays, and immunoblots

For more information, see the Methods section in the Online Repository.

Flow-cytometric analysis of calcium mobilization and membrane FcεRI expression

For more information, see the Methods section in the Online Repository.

RESULTS

Discovery of C-13 and the identification of its binding cavity on Syk

We have developed the antibody displacement assay to identify small molecules able to displace the association of scFv G4G11 with Syk. Among the members of a 3000 molecule chemical library, 15 small molecules were able to compete with the binding of scFv G4G11 to Syk, and 1 compound, C-13 (Fig 1, *A*), showed the best inhibition potential, with an inhibitory concentration 50 (IC50) estimated at 4 μmol/L (Fig 1, *B*). This is in agreement with the value of 4.8 μmol/L obtained by the measurement of the *in vitro* affinity of C-13 for Syk (Fig 1, *B*). To understand the mechanism of action of C-13, we first mapped the binding site of G4G11 using the SPOT method.[16] A linear epitope located at the N-terminal SH2 domain of Syk, encompassing amino acids 65 to 74 and 100% conserved in mouse, rat, and human sequences, was identified.