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A novel drug-like Syk binder prevents anaphylactic shock when administered orally

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ABSTRACT

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 due to its critical position upstream to 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 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 drug-like compounds on mast cell activation were evaluated *in vitro* and *in vivo*.

Results: We discovered C-13, a small molecule that inhibits FcεRI-induced mast cell degranulation *in vitro* and anaphylactic shock *in vivo*. Importantly, C-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 two SH2 domains and the interdomain A of Syk.

Conclusion: We have isolated a new class of drug-like compounds that modulate the interaction of Syk with some of its macromolecular substrates implicated in the degranulation pathway in mast cells.

Clinical Implication: C-13 is a small molecule that bears promising anti-allergic properties when administered orally, illustrating the strong therapeutic potential of drug candidates isolated using our approach.

CAPSULE SUMMARY

We report the discovery of a drug-like compound that inhibits anaphylactic shock in mice when administered orally, and that binds to a new cavity that is localized outside the catalytic domain of the tyrosine kinase Syk.

Keywords: Allergy and Inflammation; Anaphylactic shock; Degranulation; Drug-like compound; Mast cells/basophils; Tyrosine kinase Syk.

Abbreviations:

ADA: antibody displacement assay

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

PLC- γ : phospholipase C γ

PSA: passive systemic anaphylaxis

PTK: protein tyrosine kinase

scFv: single chain variable domain

SLP-76: Src homology 2 (SH2) domain-containing leukocyte protein of 76 kD.

INTRODUCTION

The PTK 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 ITAM motifs that form a binding site for Syk which is subsequently activated.

Active Syk then phosphorylates many substrates including the adapter proteins LAT, SLP-76, and Vav, leading to the activation of several signaling pathways, such as those of 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 positively regulate FcεRI signaling ³, suggesting that it could be an excellent target for treating allergic disorders. Pharmacological inhibitors of Syk kinase activity bearing therapeutic potential have been developed ⁴. However, Syk is widely distributed in different cell types, and one wants to reduce the risk of unwanted consequences of inhibiting the catalytic activity of this kinase on various physiological functions such as cell differentiation, adhesion and proliferation ^{5, 6}. To this aim, one must develop new approaches 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 scFv G4G11 was isolated from a combinatorial library screened against a recombinant protein containing Syk SH2 domains ⁸. We hypothesized that G4G11 binds to a region of Syk that interacts with partner(s) 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 drug-like compounds that act as functional mimics of G4G11. We identified 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 *in vivo* with passive cutaneous and passive systemic anaphylaxis in BALB/c mice.

METHODS

Chemicals and antibodies. A chemical library of 3000 molecules (a diverse subset) was purchased from ChemBridge, Inc. (San Diego, CA). Stock solutions of small molecules were prepared at 10 mM in DMSO, 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 for the irrelevant chemical (ChemBridge ID number 5522980) which were prepared in DMF. All reagents unless otherwise mentioned were from Sigma. The hapten dinitrophenyl (DNP) was purchased from Calbiochem. Antibody reagents are described in the Methods section in the Online Repository.

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

Recombinant GST:Syk 6-242 fusion protein⁸ was immobilized on an ELISA plate at final concentration of 10 $\mu\text{g ml}^{-1}$. For the screening of the chemical library, small molecules diluted in PBS at final concentration of 10 μM were added to the wells for one hour at RT, before adding myc-tagged scFv G4G11 at final concentration of 100 nM for one additional hour. The binding of G4G11 to Syk was evaluated by adding HRP-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 GST:Syk 6-242 protein, and the binding of G4G11 to the mutants was evaluated in the presence of 5 μM C-13.

Cells, culture conditions and functional assays. The mouse IgE anti-DNP monoclonal antibody 2682-I was used as hybridoma culture supernatants which contained 1 $\mu\text{g ml}^{-1}$ IgE. Femoral bone marrow cells were collected and cultured in Opti-MEM medium (Gibco) supplemented with 10% fetal bovine serum and 4% supernatant of X63 transfectants secreting

murine IL-3. RBL-2H3 rat basophilic leukaemia cells (ATCC) were maintained as monolayer cultures in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco).

Measurements of β -hexosaminidase release in RBL-2H3 cells were performed as described ⁷, except that after 12-16 h incubation with anti-DNP IgE ($0.5 \mu\text{g ml}^{-1}$), cells were incubated for 90 min at 37°C in RPMI medium supplemented with the indicated concentrations of C-13 or DMF (0.25%). Cells were challenged for 45 min with DNP-BSA (50 ng ml^{-1}) or ionomycin ($1.5 \mu\text{M}$). BMMCs were incubated for 1 h at 37°C with anti-DNP IgE (100 ng ml^{-1}). They were then incubated with C-13 ($3 \mu\text{M}$) or DMF (0.3%) for 3 h at 37°C , and challenged with various concentrations of DNP-BSA. β -hexosaminidase release was measured 10 min later, and TNF- α was titrated by cytotoxicity assay on L929 cells as described ¹⁰, 3 h after challenge.

Induction of anaphylaxis. Female BALB/c mice (6-8 week old) were purchased from Charles River and kept at the IRCM animal house under pathogen-free conditions. Protocols for IgE-dependent PSA and PCA were carried out as described ¹¹. Briefly, mice were injected intravenously with $100 \mu\text{g}$ IgE (SPE-7, Sigma) in $200 \mu\text{l}$ PBS for PSA, or intradermally with 25 ng IgE in $10 \mu\text{l}$ PBS for PCA, and challenged 24h later with intravenous injection of 1 mg DNP-KLH in 2% Evans blue. C-13, irrelevant chemical or vehicle were administered either orally (PSA) in $200 \mu\text{l}$ carboxymethylcellulose 1%, or locally (PCA) to the ear skin in acetone/olive oil (4:1), 1 hour prior to the challenge. Animals were sacrificed 20 min after the challenge. Ear were removed, minced and Evans blue was extracted by overnight incubation in formamide at 80°C . For temperature measurements in PSA, C-13 (100 mg kg^{-1}) or vehicle were administered orally, 3 hours prior to the challenge performed in the absence of Evans blue. Temperature was monitored using an electronic thermometer with a rectal probe (YSI, Yellow Springs, OH) before challenge, and for 60 minutes afterwards, before sacrifice. Absorbance was measured at 610 nm.

Structural studies. Druggable pockets were predicted with Q-SiteFinder ¹² and ICM ¹³. C-13 was docked using LigandFit ¹⁴ and Surflex ¹⁵. The top 20 poses were analyzed and one consensus pose is presented Fig. 1A. Images were generated with PyMol.

Statistical analysis. Averaged numeric data are expressed as means \pm standard deviations. Student's *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 Fc ϵ RI expression. For more information, see the Methods section in the Online Repository.

RESULTS

Discovery of compound 13 and the identification of its binding cavity on Syk

We have developed the ADA 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 one compound called hereafter C-13 (Fig. 1A) showed the best inhibition potential with an IC₅₀ estimated at 4 μ M (Fig. 1B). This is in agreement with the value of 4.8 μ M obtained by the measurement of the *in vitro* affinity of C-13 for Syk (Fig. 1B). To understand the mechanism of action of C-13, we first mapped the binding site of G4G11 using the SPOT method¹⁶. A linear epitope located at the N-terminal SH2 domain of Syk, encompassing amino acids 65-74 and 100% conserved in mouse, rat and human sequences was identified.

Based on this data and on the known 3D structure of Syk SH2 domains-ITAM peptide complex¹⁷, we used computational approaches to search for a putative binding site able to accommodate C-13. A candidate pocket located next to the epitope of G4G11 was identified (Fig. 1A). Structural analysis indicated that residues Ser 9, Gln 43, Phe 51, Arg 68, Glu 121 and Glu 155 could be involved in ligand binding, and could be mutated without damaging the 3D fold of the protein. For further experimental validation of the pocket, the six amino-acids were individually mutated and Syk mutants were subjected to the ADA. Residues Arg 68, Glu 121 and Glu 155 were found to contribute significantly to the interaction with the small molecule, as their mutation abrogated the inhibition due to C-13, while the binding of scFv G4G11 was maintained (Fig. 1C). These data confirmed that the binding cavity of C-13 on Syk was localized in the near vicinity of the G4G11 binding site.

Fc ϵ RI-induced mast cell activation

To examine functional similarities with G4G11, we explored the biological effects of C-13 on mast cell activation. The incubation of RBL-2H3 cells with C-13 did not affect FcεRI-induced Syk phosphorylation and kinase activity (Fig. 2A) and, accordingly, the overall tyrosine phosphorylation of total cellular proteins known to be mainly dependent on Syk was normal (Fig. 2B, C). Similar to the intrabody G4G11, C-13 inhibited FcεRI-induced Btk phosphorylation and its kinase activity (Fig. 2A) and the phosphorylation of PLC-γ1 and PLC-γ2, the two PLC-γ isoforms expressed in mast cells (Fig. 2A, C). The PTK Lyn phosphorylates both Syk and Btk leading to their full activation and to the subsequent phosphorylation of PLC-γ¹⁸. As C-13 did not affect FcεRI-mediated activation of Lyn (Fig. 2A), we concluded that the reduced phosphorylation of Btk and PLC-γ could be due to a defect in their proper localization to the vicinity of upstream PTKs.

Analysis of Fyn- and Lyn-mediated signaling pathways

In mast cells, the Fyn/Gab2/PI3K signaling pathway leads to the activation of PI3K and the generation of PI-3,4,5-P₃ that recruits a number of pleckstrin homology (PH) domain-containing proteins, including Btk and PLC-γ to the plasma membrane¹⁹. The analysis of the phosphorylation of Akt, a marker of PI3K activity, indicated that C-13 did not affect the Fyn mediated pathway (Fig. 2B, C), suggesting that the reduced phosphorylation of Btk and PLC-γ was not due to a defect in their membrane localization, known to be essential for calcium responses²⁰.

The recruitment of Btk and PLC-γ to the membrane requires also the canonical Lyn/Syk/LAT/SLP-76 signaling pathway. The phosphorylation of LAT by Syk leads to the translocation of SLP-76 to the LAT-organized complex²¹, where it co-localizes with Syk²². This localization permits Syk to phosphorylate N-terminal tyrosines in SLP-76²³ which

become binding sites for Vav, Nck and Btk. LAT and SLP-76 (through its prolin-rich domain recruiting PLC- γ) localize PLC- γ cooperatively to this membrane complex, allowing for phosphorylation and activation of PLC- γ by Btk²⁴ and/or Syk²⁵. The use of phospho-specific antibodies showed that C-13 inhibited the phosphorylation of SLP-76, but increased the phosphorylation of LAT in a dose-dependent manner (Fig. 2A). We hypothesized that the inhibition of phosphorylation of SLP-76 could allow more LAT to interact with Syk, thus causing enhanced phosphorylation. The reduced phosphorylation of SLP-76 was not due to a defect in its recruitment to LAT, and resulted in a defect in the co-localization of Btk and to a lesser extent that of Vav with SLP-76 (Fig. 2A). Nevertheless, the phosphorylation of Vav known to be independent of its recruitment to SLP-76²⁶ was not inhibited (Fig. 2A).

MAPK activation

The association of SLP-76 with Vav and/or Nck contributes to the optimal activation of MAP kinases in mast cells²⁷. We found that C-13 mildly affected the activation of MAP kinases (evaluated through their phosphorylation): high concentration of C-13 decreased ERK1/2 phosphorylation, while p38 and JNK phosphorylations were normal (Fig. 2B, C).

Calcium flux and degranulation

The binding of Btk and Vav to SLP-76 is critical for the regulation of the activity of PLC- γ at the membrane, calcium mobilization and granule exocytosis^{27, 28}. We found that the association of PLC- γ with LAT was inhibited by C-13 in a dose-dependent manner (Fig. 2A). Consistent with the defect in PLC- γ 1 and PLC- γ 2 phosphorylation, mast cells displayed reduced calcium flux amplitude in response to Fc ϵ RI ligation (Fig. 3A), and the early and the late Fc ϵ RI-induced allergic responses in BMMC and in the RBL-2H3 cell line were impaired

in a dose-dependent manner, based on the measure of β -hexosaminidase release and TNF- α secretion (Fig. 3B, C, D). Our results also showed that C-13 had no toxic effect on mast cells. Indeed, neither ionomycin-induced degranulation (Fig. 3B) nor viability of BMMCs (Fig. E1) was detectably affected by C-13 treatment. Moreover, the observed defects in mast cell activation were not due to a reduced surface expression of Fc ϵ RI, because flow cytometry analysis indicated that cells incubated with C-13 expressed similar levels of Fc ϵ RI compared to control cells (Fig. 3E).

Passive systemic and passive cutaneous anaphylaxis

To extend these observations on mast cell functions *in vivo*, we tested the effects of the oral administration of C-13 on PSA and on PCA induced in BALB/c mice by the administration of DNP-specific IgE molecules followed by intravenous challenge with the hapten DNP-KLH. The intensity of systemic anaphylaxis was assessed by monitoring both the decrease of body temperature and the increase of vascular permeability following antigen administration. Upon the oral administration of C-13 (and before antigen challenge), animals appeared healthy with no overt sign of toxicity. A single oral administration of 100 mg/kg C-13 inhibited hypothermia and hastened animal recovery (Fig. 4A). Based on the quantification of Evans blue dye extravasation, we determined that C-13 inhibited the increase in vascular permeability with an IC₅₀ estimated at 110 mg/kg (Fig. 4B and 4C). C-13 showed also an inhibitory effect on PCA with an IC₅₀ estimated at 25 μ M (Fig. 4D).

Other Syk-dependent responses

Finally, we determined to which extent *in vivo* effects of C-13 were restricted to mast cells. Our data showed that at a single dose of 100 mg/kg at which C-13 efficiently inhibited anaphylaxis, the molecule did not inhibit thioglycollate-induced neutrophil

recruitment into the peritoneal cavity in the presence of *Bordetella pertussis* toxin (Fig. E2A), which has been shown to be fully dependent on Syk (Ref. E2). In addition, although C-13 affected anti-IgM-induced B cell proliferation *in vitro* (Fig. E2B), the oral administration of a single dose of 150 mg/kg C-13 did not affect the antibody production following immunization with a thymo-dependent antigen, which involves Syk-dependent B cell activation (Fig. E2C). Taken together, these data suggest that *in vivo*, C-13 displays a selective effect on mast cell-dependent responses.

DISCUSSION

We report here the discovery of the drug-like compound C-13 which inhibits anaphylactic shock when administered orally. C-13 binds to a novel cavity located at the interface between the two SH2 domains and the interdomain A of Syk (Fig. 1). C-13 binding pocket forms a rather unique interaction area that is specific to Syk, and does not correspond to a known binding site of a physiological ligand of Syk such as the dually tyrosine phosphorylated ITAM peptide (Fig. 1A). The detailed molecular mechanism of the inhibitory effects of C-13 on mast cell functions is under investigation. At this stage, our data suggest that C-13 inhibits the interaction of Syk with some of its macromolecular substrates, either directly because part of C-13 occupies a surface where a Syk partner could make direct contact, and/or through an allosteric effect.

Our biochemical studies in mast cells indeed showed that C-13 inhibits the FcεRI-dependent phosphorylation of the Syk substrate SLP-76 at tyrosine residues that contribute to its adapter function for binding and/or stabilization of Btk, PLC-γ and Vav to the macromolecular signaling complex formed with LAT^{22, 28-30} (Fig. 2). This is affecting the phosphorylation and the catalytic activity of Btk and the turnover of PLC-γ to the proximity of Syk and/or Btk for its full phosphorylation that is needed for a sustained calcium flux and exocytosis³¹⁻³⁵. Indeed, C-13 inhibited the early (β-hexosaminidase release) and the late (TNF-α secretion) mast cell responses induced by FcεRI aggregation, with an IC₅₀ estimated at 2 μM (Fig. 3).

Importantly, the oral administration of a single dose of C-13 inhibited IgE-induced Passive Systemic Anaphylaxis, with an IC₅₀ estimated at 110 mg/kg (Fig. 4), emphasizing the promising anti-allergic properties of this compound. The *in vivo* inhibitory effects of C-13 appeared to be restricted to mast cells. Indeed, a single oral administration of 100 mg/kg C-13

which inhibited anaphylaxis, did not affect Syk-dependent thioglycollate-induced neutrophil recruitment into the peritoneal cavity in the presence of *Bordetella pertussis* toxin (Fig. E2A). Moreover, although *in vitro* BCR-dependent B cell proliferation was dose-dependently inhibited by C-13 (Fig. E2B), antibody responses of mice immunized with a thymo-dependent antigen was not affected by the oral administration of 150 mg/kg C-13 (Fig. E2C). C-13 therefore does not affect other Syk-dependent non-mast cell-dependent *in vivo* responses at doses and time scale at which it can inhibit a severe allergic response.

Considering the absence of any apparent toxic effects following oral or local administration of C-13 over a period ranging from one hour to 12 days, we believe that C-13 may represent the first member of a new family of orally available Syk inhibitors, and pharmacologically active anti-inflammatory drugs. The drug screening approach described here constitutes a generic platform in which the primary use of antibodies points out to the domains of the target that bear a therapeutic potential, thus facilitating the design of chemicals (through *in silico* and/or *in vitro* screening) able to act as functional mimics of the antibody, and as potential protein-protein interaction inhibitors. Further, we show that these small compounds can induce the desired response in cellular and animal models supporting the concept of replacing large macromolecules difficult to administrate by orally available small organic molecules.

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FIGURE LEGENDS

Figure 1. Binding of C-13 to Syk *in vitro*. **A.** The C-13 pocket (mesh representation) was predicted through theoretical means and validated by mutagenesis. Figure illustrates C-13 docked into this newly identified Syk cavity located next to scFv G4G11 epitope. **B.** Binding of G4G11 to Syk in ADA in the presence of C-13 (■). Binding of C-13 to Syk measured using fluorescence spectroscopy (○). **C.** Binding of G4G11 to Syk mutants in ADA. Data shown are representative of at least 2 experiments. Significant inhibition with C-13 versus DMF: **P < .01.

Figure 2. C-13 impairs FcεRI-induced mast cell activation. **A.** Immunoprecipitations performed on RBL-2H3 cell lysates were analyzed by immunoblotting with the indicated antibodies. *In vitro* kinase activities of Syk, Btk and Lyn immunoprecipitates were examined. **B.** RBL-2H3, and **C.** BMMCs cell lysates were subjected to electrophoresis and proteins were analyzed by immunoblotting. These representative data are from at least 2 experiments.

Figure 3. C-13 inhibits FcεRI-mediated calcium release and degranulation. **A.** FACS analysis of IgE-mediated calcium flux in RBL-2H3 cells. **B.** β-hexosaminidase release measurement in RBL-2H3 cells. **C.** β-hexosaminidase release, and **D.** TNF-α titration in BMMCs. **E.** FACS analysis of surface expression of FcεRI in RBL-2H3 cells. (0 = 0.25% DMF). These representative data are from 3 experiments. Significant inhibition with C-13 versus DMF: **P < .01 and *P < .05.

Figure 4. *In vivo* studies on BALB/c mice. **A-C.** PSA response. **A.** Temperature change, **B.** Quantification of Evans blue extravasation, **C.** Photograph representing dye extravasation following oral administration of 130 mg/kg C-13 or irrelevant chemical (IR); carrier (T); non-treated animal (NT). **D.** PCA response: quantification of Evans blue extravasation. 4-5 mice were used per condition. Data shown are representative of 3 experiments. Significant inhibition with C-13 versus irrelevant chemical: $**P < .01$; versus carrier+DNP-KLH: $*P < .05$.