Specific Inhibition of Spleen Tyrosine Kinase Suppresses Leukocyte Immune Function and Inflammation in Animal Models of Rheumatoid Arthritis

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Received September 26, 2011; accepted October 25, 2011

ABSTRACT

Based on genetic studies that establish the role of spleen tyrosine kinase (Syk) in immune function, inhibitors of this kinase are being investigated as therapeutic agents for inflammatory diseases. Because genetic studies eliminate both adapter functions and kinase activity of Syk, it is difficult to delineate the effect of kinase inhibition alone as would be the goal with small-molecule kinase inhibitors. We tested the hypothesis that specific pharmacological inhibition of Syk activity retains the immunomodulatory potential of Syk genetic deficiency. We report here on the discovery of (4-(3-(2H-1,2,3-triazol-2-yl)phenylamino)-2-((1R,2S)-2-aminocyclohexylamino)pyrimidine-5-carboxamide acetate (P505-15), a highly specific and potent inhibitor of purified Syk (IC_{50} 1–2 nM). In human whole blood, P505-15 potently inhibited B cell antigen receptor-mediated B cell signaling and activation (IC_{50} 0.27 and 0.28 μM, respectively) and Fcε receptor 1-mediated basophil degranulation (IC_{50} 0.15 μM). Similar levels of ex vivo inhibition were measured after dosing in mice (Syk signaling IC_{50} 0.32 μM). Syk-independent signaling and activation were unaffected at much higher concentrations, demonstrating the specificity of kinase inhibition in cellular systems. Oral administration of P505-15 produced dose-dependent anti-inflammatory activity in two rodent models of rheumatoid arthritis. Statistically significant efficacy was observed at concentrations that specifically suppressed Syk activity by ~67%. Thus specific Syk inhibition can mimic Syk genetic deficiency to modulate immune function, providing a therapeutic strategy in P505-15 for the treatment of human diseases.

Introduction

Spleen tyrosine kinase (Syk) is broadly involved in regulating leukocyte immune function, principally by facilitating cellular activation in response to receptor engagement of antigen or immune complex. Receptors that use Syk for signal transduction include the B cell antigen receptor (BCR), Fc receptors, integrins, and members of the lectin and selectin families (Turner et al., 2000; Mócsai et al., 2002; Rogers et al., 2005; Zarbock et al., 2008). In B cells, signaling through the BCR regulates cellular activation and promotes survival and clonal expansion. Fc receptors initiate immunoglobulin-mediated inflammatory responses by basophils and mast cells (via FcεR and FcγR), and by eosinophils, neutrophils, dendritic cells, and macrophages (via FcγRa). Syk is additionally implicated in integrin signaling, which is responsible for neutrophil effector functions (Mócsai et al., 2002) and leukocyte rolling in vivo (Fromholt et al., 2007) as well as in signaling via the triggering receptor expressed on myeloid cells (TRREM-1), which is involved in osteoclast bone resorption (Fodor et al., 2006). The platelet collagen receptor glycoprotein VI also requires Syk for signal transduction after collagen binding (Poole et al., 1997). Recently, platelets were implicated in the etiology of human rheumatoid arthritis (RA), and glycoprotein IIb/IIIa receptor antagonists are currently in clinical trials for the treatment of RA.
tein VI was identified as the key receptor mediating inflammatory activity in mice (Boillard et al., 2010). Each of these Syk-dependent functions in various cell types is likely to be important in the etiology of inflammatory and autoimmune diseases.

Syk is therefore considered an important target for controlling diseases involving immune cells. To test the hypothesis that Syk is required for inflammatory responses to auto antibodies, fetal liver cells from Syk(−/−) or wild-type mice were adoptively transferred into lethally irradiated wild-type mice followed by injection of arthritogenic K/B×N serum (Jaks et al., 2010). Syk-deficient cells were incapable of mounting an inflammatory response in this model. Although the experiments provide in vivo evidence for the requirement of Syk in mediating inflammation, they do not demonstrate a specific requirement for the kinase activity of this protein. There are examples where genetic deficiency of a kinase was not phenotypically copied by defective kinase activity, suggesting that nonkinase-dependent adapter/scaffolding functions rather than enzymatic activity played an essential role. Selective inhibition of Zap70 kinase activity in mice did not lead to defects in Rap1 activation or integrin-mediated cell adhesion, as was observed with Zap70 genetic deficiency (Au-Yeung et al., 2010). Likewise, knockdown of interleukin 1 receptor associated kinase 1 by small hairpin RNA induced cell death in a subset of non-Hodgkin’s lymphoma cell lines, a phenotype that was completely rescued by expression of a kinase-dead mutant of this protein (Ngo et al., 2011). The requirement for Syk activity for inflammatory responses has been tested by using the inhibitor R406/R788 (Braselmann et al., 2006). Treatment with this kinase inhibitor was inhibitory in rodent models of RA (Braselmann et al., 2006; Pine et al., 2007) and demonstrated therapeutic benefit in phase II clinical trials of RA (Weinblatt et al., 2007) and demonstrated therapeutic benefit in rodent models of RA (Braselmann et al., 2006; Pine et al., 2007) and demonstrated therapeutic benefit in phase II clinical trials of RA (Weinblatt et al., 2008). However, the specific contribution of Syk inhibition to the efficacy observed in these studies is not clear because R406/R788 inhibits multiple kinases (Braselmann et al., 2006).

Here, we report on the discovery and characterization of (4-(1S,2R,3S)-3-ethyl-2-phenylamino)-2-((1R,2S)-2-amino-cyclohexylamino) pyrimidine-5-carboxamide acetate (P505-15, PRT062607), a novel, highly specific, and potent orally available small-molecule inhibitor of Syk. This compound provides a tool with which to test the hypothesis that specific pharmacological inhibition of Syk activity is sufficient to modulate leukocyte immune function and ameliorate inflammation in vivo. We demonstrate here the specificity of P505-15 inhibitory activity in purified enzyme and cellular assays. Furthermore, we estimate Syk-dependent pharmacodynamic effects of the inhibitor in human and rodent blood and test for efficacy in two rodent models of RA. We conclude from these data that specific inhibition of Syk function by P505-15 is sufficient to suppress inflammation in vivo. P505-15 therefore represents a reasonable therapeutic strategy for treating diseases involving aberrant immune function.

**Materials and Methods**

**Cell Lines and Reagents.** The human non-Hodgkin’s lymphoma B cell lines SUDHL4 (ACC495; Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and Ramos (CRL-1596; American Type Culture Collection, Manassas, VA) were maintained in RPMI media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (American Type Culture Collection) and penicillin/streptomycin (Invitrogen). The Ba/F3 mouse pro-B cell lines stably expressing either JAK1, JAK2, JAK3, Tyk2, Syk, or Zap70 were obtained from Advanced Cellular Dynamics (San Diego, CA) and maintained in RPMI media supplemented with 10% HyClone fetal calf serum (Thermo Fisher Scientific, Waltham, MA) and 0.5 µg/ml puromycin (Mediatech, Manassas, VA). Antibodies used in these studies included polyclonal goat F(ab’/2 anti-human IgM (BioSource International, Camarillo, CA), goat anti-human IgD and anti-human IgE (Bethyl Laboratories, Montgomery, TX), goat anti-mouse IgG antisera (E Biosciences, San Diego, CA), and goat serum (St. Louis MO) as an isotype control; rabbit anti-human Syk, anti-human phospho-Syk (Tyr352), anti-human BLNK, anti-human phospho-BLNK (Tyr84), anti-human phospho-ERK (Tyr204), and recombinant full-length Syk (glutathione transferase fusion protein) were obtained from Cell Signaling Technology (Danvers, MA). Phycoerythrin (PE)-conjugated mouse anti-human phospho-Zap70 (Tyr319/Syk (Tyr352), PE-conjugated anti-human CD69, fluorescein isothiocyanate, PE, PerCP, and allopheophycin (APC)-conjugated anti-human CD19 and PerCP-conjugated anti-mouse CD45R/B220 were obtained from BD Biosciences (San Jose, CA). APC-conjugated goat F(ab’/2 anti-rabbit IgG (H+L) was obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Syk substrate peptide was obtained from Multiple Peptide Systems (San Diego, CA). Streptavidin-conjugated APC and europium-conjugated antiphosphotyrosine antibody were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA).

**Synthesis of Syk Inhibitors.** Extensive structure-activity relation studies identified the highly potent and specific Syk inhibitor P505-15. P505-15 was purified by reverse-phase high-performance liquid chromatography and characterized by mass spectrometry and NMR analysis for structural identity and confirmation.

**Purified Kinase Assays.** Potency of Syk inhibition was determined by using a fluorescence resonance energy transfer (FRET) assay (Biazzo-Ashnault et al., 2001). The extent of substrate phosphorylation by Syk was measured in the presence of various P505-15 concentrations. Syk activity was determined by a fluorescent antibody specific for phosphorylated tyrosine by using the increase of fluorescence by using the increase of substrate phosphorylation by Syk was measured in the presence of various P505-15 concentrations. Syk activity was determined by a fluorescent antibody specific for phosphorylated tyrosine by using the increase of

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Intracellular Phospho-Flow Cytometry in Whole Blood.

Blood was collected from the antecubital vein of subjects who gave written informed consent to the protocol (approved by the Human Subjects Committee of Portola Pharmaceuticals Inc.). Human or mouse blood was collected directly into lymphopiled heparin-containing tubes. Human blood (200 μl) was pretreated with vehicle control or P505-15 (seven concentrations for dose response), as indicated, for 30 min at 37°C. After treatment, blood cells were then stimulated for 10 min with goat anti-human IgG or 80 nM phorbol 12-myristate 13-acetate (PMA; Sigma) to induce signaling. Stimulation was terminated by the inclusion of 2 ml of Lyse/Fix Buffer (BD Biosciences) and incubation for 10 min at room temperature. Fixed cells were then permeabilized in −20°C methanol [50% in phosphate-buffered saline (PBS)], washed, resuspended in PBS containing 1% bovine serum albumin (BSA), and stained with mouse anti-pSyk Tyr252 PE conjugate, mouse anti-human CD19 PerCP conjugate, and rabbit anti-pERK Tyr204 unconjugated. After 1-h incubation at room temperature, cells were washed once and resuspended in PBS/BSA buffer containing goat anti-rabbit polyclonal APC-conjugated secondary antibody. After 30-min incubation, cells were washed and subjected to flow cytometry by using the FACSCalibur (BD Biosciences). Compensations were set by using cells stained separately with anti-CD19 antibody conjugated to each of the three fluorophors used.

B Cell Activation and Basophil Degranulation in Human Whole Blood.

Human whole blood was collected into heparin, and 200-μl aliquots were pretreated for 30 min in a 37°C tissue culture incubator with vehicle or various concentrations of P505-15 (seven concentrations for dose response). Blood cells were activated in whole blood by incubating them with anti-human IgD antibody or 80 nM PMA for 16 h at 37°C. Blood was then stained directly with anti-CD69 PE-conjugated and anti-CD19 APC-conjugated antibodies for 30 min. Cells were fixed, and red blood cells were lysed by suspending them in fluorescence-activated cell sorting/lyse solution (BD Biosciences) for 10 min. Cells were washed in PBS/BSA and analyzed by flow cytometry.

For basophil degranulation assays, 100 μl of heparinized human blood was pretreated with vehicle or various P505-15 concentrations for 1 h, then stimulated for 20 min with either anti-human IgE (diluted 1:333 in BasoTest supplied wash buffer) or N-formyl-1-methionyl-l-leucyl-l-phenylalanine diluted 1:20 in wash buffer, and processed as detailed for the BasoTest kit (Orpegen Pharma, Heidelberg, Germany). Detection of basophil degranulation was performed as described in the supplied protocol.

Mouse Pharmacokinetic and Pharmacodynamic Relationship Studies. All animal studies were performed in strict accordance with the Institutional Animal Care and Use Committee ethical guidelines. Female BALB/c mice (Charles River, Hollister, CA) received a single oral dose of 15 or 30 mg/kg P505-15 and were anesthetized with a subcutaneous ketamine cocktail, and blood was harvested directly into lyophilized heparin-containing tubes. Human blood (200 μl) was injected intraperitoneally. Mice were orally dosed with vehicle or P505-15 b.i.d. for the duration of the study, starting just before the monoclonal antibody injection. The extent of joint and paw inflammation (sum of all four paws) was visually scored daily, and body weights were obtained every 2 days. Inflammation scores for each paw were determined based on the following scales: for hind paws, 0, no detectable swelling; 1, swelling in metatarsal phalangeal joints, an individual phalanx, or local edema, 2, swelling extends to both sides of paw but localized to only the dorsal side of a paw, and 3, swelling (severe) on all aspects of a paw, both dorsal and ventral side swelling; for front paws, 0, no detectable swelling, 1, visible reddish, mild swelling of the paw or just single digit swelling, 2, clear paw swelling or more than two digits swelling, and 3, whole paw swelling (severe), including both dorsal and ventral sides. Ankle thickness measurements were obtained by a caliper device. At study termination, mice were anesthetized, and blood was harvested by cardiac puncture for complete blood counts and drug plasma concentration.

Rat Collagen-Induced Arthritis Model. Collagen-induced arthritis (CIA) was induced in female, 7-week old Lewis rats (Harlan, Indianapolis, IN) by subcutaneous injection of bovine collagen II (Chondrex) emulsified with incomplete Freund's adjuvant at the base of the tail (Trentham et al., 1977; Holmdahl et al., 2001). On day 10, the rats were boosted with a second subcutaneous injection. P505-15 administration was initiated when at least one hind paw was inflamed (inflammation score 1), and enrollment into a treatment group was designated as day 1 of the study for each individual animal (n = 8/dose group). Progression of disease was evidenced by increased edema and erythema of one or both ankle joints, followed by the involvement of the metatarsal and interphalangeal joints. Fully developed arthritis was observed approximately 4 to 7 days after the onset of inflammation. Hind paws (sum of two paws) were scored daily for progression of inflammation. Inflammation scores for each paw were determined based on the following scale: 0, normal; 1, mild, but definite redness and swelling of the ankle possibly limited to individual digits; 2, moderate redness and swelling of ankle; 3, severe redness and swelling of the entire paw including digits; and 4, maximally inflamed limb with involvement of multiple joints. Ankle thickness measurements were obtained by a caliper device. At study termination, rats were anesthetized, and blood was harvested by cardiac puncture for complete blood counts and drug plasma concentration.

Histopathology Scores. The hind feet of study animals as well as naive controls were harvested and placed in 10% buffered formalin for histopathological evaluation. Microscopy was performed in a blinded fashion by using the Olympus (Tokyo, Japan) BX510 light microscope with either the UPLanApo Olympus 10× NA 0.4 or 4× NA 0.16 objectives. Images were captured by using Nikon (Tokyo, Japan) DXM1200 or Olympus DP21 cameras and manufacturer-associated software. Rat tissues were evaluated by using a modified Mankin score (Mankin, 1971) as described previously (Pine et al., 2007). Mouse tissues were scored (0, no change to 4, severe) for the presence of intra-articular neutrophils and fibrin, periosteal hyperplasia, chondrocyte necrosis, dermal edema, and osteolysis.

Data Analysis and Statistics. The effective concentrations at 25, 50, and 90% of the maximal response were determined by nonlinear regression of the data by using the following equation:

\[
f(x) = c + \frac{d - c}{1 + \exp(b \log(x) - \log(e))}
\]  

where d is the maximal response, c is the minimal response, b is the slope, and e is the inflection point of the curve. In the present study c was assumed to be 0, and d was set for all curves at 100. The data were analyzed by using robust regression as implemented in the drc package of the statistical analysis software R (http://www.r-project.org/) (Ritz and Streibig, 2005). Statistical evaluation of P505-15 effect in rodent inflammation
models was performed by using a $t$ test at the terminal time point and two-way analysis of variance for repeated measures over the entire data set. Data were graphed by using Prism (GraphPad Software Inc., San Diego, CA).

### Results

**P505-15 Is a Potent and Selective Inhibitor of Syk in Purified Kinase Assays.** We synthesized a series of potent and highly specific ATP-competitive small-molecule inhibitors of Syk, from which P505-15 was identified as a candidate for clinical development (chemical structure shown in Fig. 1a). The potency of P505-15 against its target kinase Syk was initially tested in two different purified kinase assays (Fig. 1b). Using a FRET assay, half-maximal Syk inhibition required $6 \pm 0.2$ nM (mean $\pm$ S.E.M.). Similar potency was observed when tested in a radioactive enzyme assay, with a resulting Syk IC$_{50}$ of $2.1 \pm 0.4$ nM (mean $\pm$ S.E.M.). Specificity for Syk was determined by screening P505-15 against a panel of 270 independent purified kinases at 50 and 300 nM in the radioactive-purified enzyme assay. The percentage of inhibition relative to vehicle control is shown as a heat map (Fig. 2a); no inhibition is represented as green, increased blending with red indicates increased percentage of inhibition of kinase activity with red representing 100% inhibition. To control for minor lot-to-lot variability, a single lot of P505-15 was used for head-to-head comparison of potency between Syk and other kinases. As depicted in Fig. 2a, P505-15 is highly Syk-specific when tested at 50 nM (top row), which was 50-fold above its Syk IC$_{50}$ (Fig. 2b; Coffey et al., 2010). When retested at 300 nM (300-fold above its Syk IC$_{50}$), P505-15 inhibited Syk and eight additional kinases by $\geq 80\%$ (Fig. 2b, bottom row). IC$_{50}$ values for each of these additional kinases are presented in Fig. 2b. The second most potently inhibited kinase (Fgr) required an 81-fold higher concentration for half-maximal inhibition. Lyn, the major Src family member expressed in B cells, was inhibited 192-fold less potently than Syk. P505-15 also showed substantially weaker inhibitory potency against

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<th>Assay</th>
<th>[ATP] (µM)</th>
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<tr>
<td>FRET</td>
<td>100</td>
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<td>Radioactive Enzyme Assay</td>
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**Fig. 1.** P505-15 is a potent inhibitor of Syk. a, the chemical structure of P505-15. b, potency of Syk inhibition in purified kinase assays. Replicate data are reported (mean $\pm$ S.E.M.) for the FRET assay ($n = 4$) and radioactive enzyme assay ($n = 8$).
the three kinases most homologous to Syk; namely, focal adhesion kinase, protein tyrosine kinase 2, and Zap70 (Fig. 2b).

Specific Inhibition of Syk Suppresses BCR Signaling in Cell Lines. Syk inhibition in SUDHL4 cells was assessed by measuring BCR-mediated induction of BLNK phosphorylation (pBLNK Tyr84), a direct measure of Syk activity, as well as the induction of Syk phosphorylation (pSyk Tyr352), a measure of Lyn activity. As shown in Fig. 3a, P505-15 inhibited BLNK Tyr84 phosphorylation in a concentration-dependent manner, whereas no inhibition of Lyn activity was observed at concentrations as high as 2.5 µM. As expected for a highly specific Syk inhibitor, P505-15 selectively inhibited the proliferation of the Syk-transformed cell line, but had no antiproliferative effect on the other cell lines at concentrations up to 2 µM (Fig. 3a). By comparison, the nonselective kinase inhibitor staurosporine inhibited proliferation in all six cell lines. P505-15 inhibited Ba/F3 TEL-Syk proliferation with an IC50 of 120 nM ± 5 (mean ± S.E.M.; Fig. 4b), which was consistent with inhibitory activity observed in other cellular systems (SUDHL4 and Ramos).

P505-15 Specifically Inhibits Syk-Dependent Leukocyte Signaling and Activation in Human Whole Blood. The effect of Syk inhibition by P505-15 on BCR signaling and B cell activation was tested in human whole blood, enabling a more accurate representation of the potency of Syk inhibition that may be achieved in circulation after oral dosing. As shown in Fig. 5a, P505-15 suppressed Syk-mediated pERK Tyr204 (mean IC50 ± S.E.M.; 0.27 µM ± 0.02) and cellular activation of B cells (CD69; mean IC50 ± S.E.M.; 0.28 µM ± 0.03) but did not inhibit Lyn-dependent signaling to pSyk Tyr352 upon BCR ligation. Inhibition of Syk activity correlated well with suppression of B cell activation. To confirm the Syk

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**Fig. 3.** Specific inhibition of BCR-induced Syk signaling in B cells. a, SUDHL4 cells were left unstimulated (NS) or stimulated with anti-IgM (anti-BCR) in the presence of vehicle control (0) or the indicated concentrations of P505-15. Western blot analyses of whole-cell lysates were then performed to evaluate Syk kinase activity (pBLNK Tyr84 and total BLNK; top) and Lyn kinase activity (pSyk Tyr352 and total Syk; bottom). A representative experiment is shown from three replicates. b, the bar graph represents mean ± S.E.M. of pBLNK Tyr84 as determined by densitometry of Western blot analyses (n = 3).

**Fig. 4.** P505-15 selectively inhibits proliferation of Syk-dependent B cell lines. a, P505-15 was tested for antiproliferative activity in a panel of B cell lines transformed with the individual kinases shown. Data represent mean percentage inhibition ± S.D. of cellular proliferation in the presence of 2 µM P505-15 or the multikinase inhibitor staurosporine (n = 7; experiments performed in duplicate). b, concentration-effect relationship of P505-15 incubated with Ba/F3 TEL-Syk B cells. Data (mean ± S.E.M.) are plotted as the extent of proliferation normalized to vehicle control. The IC50 (0.12 µM; represented by the dashed line) and 95% confidence interval (0.105–0.127 µM; horizontal bar) are shown.
specificity of this effect, whole blood was stimulated with anti-IgD (anti-BCR) or PMA to induce Syk-dependent or -independent signaling and functional activation of B cells. As shown by representative fluorescence-activated cell-sorting scatter plots in Supplemental Fig. 1b, 2 µM P505-15 potently suppressed BCR-dependent, but not PMA-dependent, signaling and activation in B cells in whole blood. Representative lymphocyte and B cell gating is shown in Supplemental Fig. 1a.

We also tested the specificity of Syk inhibition by P505-15 in a second source of primary cells. Basophils in whole blood were stimulated with anti-IgE antibody to cross-link the FceRI (Syk-dependent signaling). Degranulation was measured by up-regulation of cell surface CD63. Incubation with P505-15 inhibited basophil degranulation after FceRI cross-linking (Fig. 5b), mean IC50 ± S.E.M., 0.15 ± 0.01 µM. The inhibition of FceRI-induced degranulation was specific, because N-formyl-L-methionyl-L-leucyl-L-phenylalanine-induced basophil degranulation was not affected by 1 µM P505-15, the highest concentration tested (see Supplemental Fig. 2b). Supplemental Fig. 2a depicts the forward by side scatter plot of peripheral blood cells, from which low-IgE expressing cells were gated out, revealing the IgE-high-expressing basophils.

Potent and Reversible Syk Inhibition by P505-15 after Oral Dosing in Mice. To determine the ability of P505-15 to suppress Syk signaling in vivo and test its affinity for murine Syk, we evaluated the compound in mice. Blood from orally dosed mice was tested for plasma concentration of P505-15 and ex vivo BCR stimulation (via anti-mouse IgD) and measurement of Syk (pERK Tyr204) and Lyn (pSyk Tyr352) activation by phospho-flow cytometry. Demonstrating a rapid onset of action, 0.5 h after oral administration of 30 mg/kg inhibitor, Syk activity was reduced by 80% and nearly completely suppressed for the next 5 h relative to vehicle control-treated mice (Fig. 6a, black bars). Syk activity remained more than 60% suppressed for the first 8 h post-dosing, returning to levels of vehicle control treatment by 24 h. At the lower dose of 15 mg/kg, more than 50% suppression of Syk activity was observed between 2 and 6 h after oral administration of compound (Fig. 6b, black bars). In both studies, Lyn activity was not inhibited (Fig. 6, a and b, gray bars). Syk IC50 in mice was calculated to be 0.32 µM (Fig. 6c), which was consistent with data obtained from human whole blood (Fig. 5a). These data were also modeled to determine the pharmacokinetic/pharmacodynamic relationship over a 24-h period assuming 15 or 30 mg/kg oral b.i.d. dosing in mice. The model predicted that >70% Syk suppression was maintained in mice over a 24-h period after 30 mg/kg dosing, with an average Syk inhibition of 85.3%. At 15 mg/kg, Syk inhibition ranged from 7.5% (Cmin) to 78.4% (Cmax) with an average inhibition of 67% over 24 h. Sustained suppression of Syk activity was not observed after a single oral dose of 5 mg/kg (data not shown).

P505-15 Suppresses Inflammation in Rodent Models of Rheumatoid Arthritis. Numerous studies were conducted by using both the mouse collagen antibody-induced arthritis (CAIA; n = 4) and rat CIA (n = 7) models to establish variability in the induction of inflammation. The average paw inflammation scores for vehicle-treated animals were 11.05 ± 0.84 and 6.6 ± 0.6 for the CAIA and CIA models, respectively (mean ± S.E.M.). Prednisolone was used as a positive control for CAIA, and both dexamethasone and methotrexate (as single agents) were used for CIA. Preliminary dose-ranging studies with P505-15 were conducted to establish the proper regimen for these two models. At comparable doses, the level of inhibition observed in the dose-ranging studies was similar to that seen in the dose-response studies (described below).

In the mouse CAIA model, oral administration of P505-15 resulted in an average inhibition of paw inflammation, as measured by daily scoring of inflammation compared with vehicle controls, of 12, 44, and 87% with average plasma concentration (C) average over 24 h) assessed at the end of the study of 0.38, 0.95, and 1.47 µM, respectively (Fig. 7a). A two-way repeated-measures analysis of variance was performed, revealing that all three factors (time, P505-15 concentration, and interaction term) were highly significant (p < 0.01). In the vehicle-treated group, the histopathological examination of the hind paws showed a marked chronic synovitis, with thickened joints and synovial lining and infiltration of leukocytes. In mice treated with 30 mg/kg P505-15, the damage to the joints was significantly reduced and seemed indistinguishable from normal mice. Blinded histopathology scores and hind paw ankle thickness were reduced in a dose-responsive manner (Fig. 7b) and correlated with
Inflammation scores. Both measurements were statistically significantly decreased ($p < 0.05$) in the 15 and 30 mg/kg dose groups compared with the vehicle control group. Compared with vehicle-treated controls there were no differences in body weight, hematological parameters, bone marrow cellularity, or small intestine histopathology in any of the P505-15-treated groups. Images of histology sections are presented in Fig. 7, c–j. Whereas extensive neutrophil infiltration and tissue damage were observed in vehicle control-treated mice (Fig. 7, g and h), joint structure similar to naive controls (Fig. 7, i and j). Photomicrographs of tarsal joints from mice treated with vehicle (Fig. 7, c and d) demonstrate intra-articular neutrophils and fibrin (+) with primarily neutrophils in the surrounding stroma (¥). In Fig. 7e, loss of articular cartilage with an irregular hyaline cartilage surface in vehicle-treated mice is shown (arrow), and higher magnification (Fig. 7f) reveals loss of cartilage to tidemark (black arrow) and pyknotic chondrocytes (red arrow). When treated with P505-15 (Fig. 7g), tarsal joints were indistinguishable from those of naive mice (Fig. 7i). A higher magnification of Fig. 7g (Fig. 7h) points to the synovial lining epithelium (white arrow), which is squamous to low cuboidal. There are no cellular infiltrates into the joint space (+) or the surrounding fascia (¥) (Fig. 7, g and h). The results demonstrate that specific inhibition of Syk by P505-15 can significantly suppress joint inflammation in a mouse CAIA model as measured by tissue swelling and inflammatory cell infiltration into the synovial tissue.

In the rat CIA model, inflammation was evident within 2 to 3 days of antigen boosting, at which time oral administration of P505-15 was initiated. We found that P505-15 treatment reduced the progression and severity of inflammation within 2 days after initiation of therapy. As shown in Fig. 8a, the high dose of P505-15 (15 mg/kg b.i.d.) completely suppressed inflammation in a majority of the animals (seven of eight), by the end of the study (mean inflammation score $\pm$ S.E.M. = 0.63 $\pm$ 1.1; $p < 0.0001$ versus vehicle). Treatment at the 10 mg/kg b.i.d. dose was also significantly efficacious and resulted in a delayed onset of disease and reduced overall inflammation (mean inflammation score $= 2.9 \pm 2.7$; $p < 0.0001$ versus vehicle). At the lowest dose, a trend toward reduction of inflammation was observed, but did not reach statistical significance. The inflammation score in the vehicle group reached a peak approximately 1 week after onset of disease with a mean score of 6.4 $\pm$ 1.8 (of a possible maximum of 8), and the arthritis symptoms did not change during the study. Hind paw sections of vehicle-treated rats exhibited extensive inflammatory infiltrate within the synovium, synovial hyperplasia, pannus formation, and erosion of articular cartilage. Glycosaminoglycan content of the cartilage was also moderately to severely reduced. Blinded histopathology scores and measurements of hind paw thickness from rats treated with P505-15 were significantly decreased ($p < 0.01$ for the 15 mg/kg dose group) and indicated a dose-responsive reduction of synovitis (Fig. 8b). At the end of the study, the average plasma concentrations for the 5, 10, and 15 mg/kg dose groups were 0.51, 0.72, and 1.34 $\mu$M, respectively. The results demonstrate that P505-15 can completely suppress
Fig. 7. P505-15 attenuates antibody-induced inflammation in a mouse model of rheumatoid arthritis. a, paw inflammation scores (mean ± S.E.M.) are depicted over time in mice dosed with vehicle or 5, 15, or 30 mg/kg P505-15 b.i.d. (12 mice per treatment group). Average plasma concentrations of P505-15 achieved in each dosing group are shown. b, the average histopathology score and average hind paw ankle thickness were determined for each mouse in a blinded fashion and plotted (mean ± S.E.M.) for each dose group. *, statistical significance, \( p < 0.05 \) by Student’s \( t \) test. c to j, joint photomicrographs of vehicle control-treated mice (c–f), 30 mg/kg P505-15-treated mice (g and h), and naive control mice (i and j), as detailed in Results. Scale bars, 100 and 400 μm, as indicated.

Discussion

Treatment for autoimmune diseases such as RA target pathways that involve leukocyte activation and inflammation. RA therapy has consisted of early and aggressive treatment for autoimmune diseases such as RA target pathways that involve leukocyte activation and inflammation. RA therapy has consisted of early and aggressive treatment with methotrexate, with the addition of tumor necrosis factor antagonists, when methotrexate response is deemed insufficient. However, 20 to 30% of patients continue to be resistant to this approach, and even in those that respond, active disease resurfaces in a number of patients. Thus, there is a need to develop safer and more effective therapeutic strategies for RA and other related autoimmune diseases.

Although specific inhibition of Syk has not yet been tested clinically for efficacy in inflammatory diseases, there is a strong rationale for the hypothesis that controlled inhibition of this kinase may be a suitable strategy. As such, the specific inhibition of Syk has the potential to control B cell antibody response to antigen as well as downstream effector functions.
Results. Scale bars, 100 and 200 µm, as indicated.

Fig. 8. Oral administration of P505-15 significantly ameliorates the severity and development of arthritis in the rat CIA model. a, the mean hind paw inflammation scores + S.E.M. as a function of time in the rat CIA model are shown. Rats were treated with vehicle or P505-15 (5, 10, or 15 mg/kg b.i.d.) at disease onset (eight rats per treatment group). Inflammation scores from both paws of each rat were evaluated daily during the 16-day treatment course. Average plasma concentrations of P505-15 (C_{pmax}) achieved in each dosing group are shown. b, the average histopathology score and average hind paw ankle thickness were determined for each rat in a blinded fashion and plotted as mean ± S.E.M. for each dose group. *, statistical significance, p ≤ 0.05 by Student’s t test. c to j, joint photomicrographs of vehicle control-treated rats (c–e), 15 mg/kg P505-15-treated rats (f–h), and naive control rats (i and j) as detailed in Results. Scale bars, 100 and 200 µm, as indicated.

of mast cells, basophils, neutrophils, eosinophils, macrophages, dendritic cells, and platelets (Mócsai et al., 2010). Targeting Syk therefore represents a multifaceted approach to modulating immune function at several points of intervention, making it a potentially unique target for drug development. Our study was designed to test the hypothesis that inhibition of Syk kinase activity was sufficient to reproducibly arrest inflammatory phenotype reported in knockout mice. We also tested whether partial suppression of kinase activity was sufficient to produce a reduction in inflammation in disease-relevant animal models.

The development of ATP-competitive small molecules that demonstrate specificity of kinase inhibition has proven to be a challenge. The catalytic domain of both tyrosine and serine/threonine kinases contain an ATP binding site. This catalytic domain is highly conserved among different kinases, so small molecules that occupy this region within a kinase of interest commonly cross-react with additional unintended targets. A relatively specific kinase inhibitor such as Gleevec is capable of binding to the less active, unphosphorylated form of the protein (Abelson tyrosine kinase) by accessing an extended ATP-binding pocket. However, pursuing a similar strategy is unlikely to be useful for identifying inhibitors of Syk. Crystallographic evidence suggests that Syk favors an active conformation, and reported structures of both staurosporine and Gleevec bound to Syk support the hypothesis that a Gleevec/Abl-like binding mode cannot be used to optimize specificity of small-molecule Syk inhibitors (Atwell et al., 2004). Through a series of chemical modifications based on studying the structure/activity relationship within a series of small-molecule Syk inhibitors, we synthesized compounds that specifically engage the catalytic domain of Syk. P505-15 was selected from this panel of compounds and, to our knowledge, represents the most potent and selective Syk inhibitor reported to date.

P505-15 demonstrated potent inhibitory activity against Syk in a variety of cellular assays, including those performed in whole blood, while maintaining specificity for this target. In animal models of arthritis, P505-15 had anti-inflammatory activity at concentrations where specificity for Syk was maintained. Syk inhibition by this compound for the treatment duration did not result in diminished leukocyte counts in animals. We interpret these data to indicate that inhibition of Syk kinase activity has potent anti-inflammatory effect in vivo. Moreover, full suppression of Syk activity was not required. Submicromolar concentrations that are predicted to result in 67% inhibition of Syk over a dosing period led to statistically significant anti-inflammatory effect in the mouse CAIA model. Similar results were obtained in the rat CIA model as well. Syk kinase inhibition by P505-15 mimics the immunomodulatory phenotype of Syk knockout observed in other rodent models of RA (Fodor et al., 2006). Thus, partial suppression of Syk signaling by P505-15 may be sufficient to ease inflammation in humans without inducing the toxicities associated with complete genetic ablation of this target (Cheng et al., 1995; Turner et al., 1995). Similar observations have been made with respect to JAK3;
gene knockouts. Genetic deficiency of this kinase leads to severe combined immuno-deficiency in mice and humans (Aringer et al., 1999), whereas partial suppression of this target by (3R,4R)-4-methyl-3-(methyl-1H-pyrrol-2,3-d-yl)pyrimidine-4-ylamino-ß-oxo-1-piperidinepropanenitrile (CP-69,550) is efficacious in human RA without significantly immunocompromising patients (Kremer et al., 2009).

In addition to autoimmune diseases, a Syk inhibitor may produce therapeutic benefit in the treatment of hematologic malignancies. Studies using primary lymphoma samples and human cell lines of non-Hodgkin’s lymphoma origin suggest a dependence of certain lymphomas on Syk signaling for survival (Davis et al., 2001, 2010; Monti et al., 2005). In chronic lymphocytic leukemia, antigen engagement of the BCR is implicated in tumor survival (Chiorazzi et al., 2005). Engagement of the BCR enhances chronic lymphocytic leukemia survival in vitro, induces the release of chemokines that facilitate interaction with stromal cells, and promotes chemotaxis toward CXCL12 and CXCL13 (Quiroga et al., 2009), suggesting that Syk mediates both intrinsic (BCR-mediated) and extrinsic survival pathways in this disease. Furthermore, intrinsic-mediated adhesion of chronic lymphocytic leukemia cells to stromal cells is blocked by Syk knockdown (Buchner et al., 2010). Thus Syk-specific inhibition by P505-15 may have broader applicability in treating certain non-Hodgkin’s lymphoma subtypes and chronic lymphocytic leukemia.

Syk, the protein tyrosine kinase, is a critical player in the diverse biological functions of this kinase. Its role in immune system development and function is now well understood (Borgwang et al., 1997). Syk is involved in the activation of innate immune cells such as dendritic cells, macrophages, and natural killer cells by engagement of signaling receptors that are linked to the immune system. Syk is also involved in the activation of adaptive immune cells such as B cells, T cells, and dendritic cells by engagement of signaling receptors that are linked to the immune system.

Authorship Contributions

Participated in research: Coffey, Hollenbach, Phillips, and Sinha.

Conducted experiments: Coffey, DeGuzman, Inagaki, Pak, Delaney, Ives, and Baker.

Contributed new reagents or analytic tools: Jia and Pandey.

Prewritten data analysis: Coffey, DeGuzman, Pak, Betz, and Baker.

Wrote or contributed to the writing of the manuscript: Coffey, Baker, Hollenbach, and Sinha.

References


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Supplemental Fig. 1: **P505-15 inhibits BCR- but not PMA-mediated B cell signaling and activation in human whole blood.**  

**a**, Flow cytometry forward by side scatter profiles of RBC-lysed human peripheral blood cells, depicting a representative lymphocyte gate. The B cell gate was established based on CD19 expression within the lymphocyte gate. 

**b**, Representative flow cytometry scatter plots gated on CD19 positive B cells. Cells were left untreated (No Stimulation) or treated with anti-BCR or PMA for 10 min in the presence of vehicle or 2µM P505-15. Intracellular phospho-flow cytometry to measure pERK Y204 (y-axis) and pSyk Y352 (x-axis) are shown in the top row. CD69 expression (y-axis) following these same stimulation and treatment conditions overnight is depicted in the bottom row.
Supplemental Fig. 2: P505-15 inhibits FcεRI- but not fMLP-mediated basophil degranulation in human whole blood. a, Forward by side scatter profiles of RBC-lysed human peripheral blood cells (left panel). Basophils were identified by IgE expression (right panel). b, Representative flow cytometry scatter plots gated on IgE positive basophils. Cells were left untreated (No Stimulation) or treated with anti-IgE or fMLP in the presence of vehicle or 1µM P505-15. CD63 expression is depicted on the y-axis, and IgE expression on the x-axis.