The novel kinase inhibitor PRT062070 (Cerdulatinib) demonstrates efficacy in models of autoimmunity and B cell cancer

Greg Coffey, Andreas Betz, Francis DeGuzman, Yvonne Pak, Mayuko Inagaki, Dale C Baker, Stanley J Hollenbach, Anjali Pandey, and Uma Sinha

Portola Pharmaceuticals, Inc., 270 E. Grand Ave, S. San Francisco CA, 94080
Running Title Page

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Correspondence Author:

Greg Coffey
Portola Pharmaceuticals
270 E. Grand Ave
South San Francisco, CA 94080
Phone: (650)246-7565
Fax: (650)246-7376
Email: gcoffey@portola.com

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Abstract

The heterogeneity and severity of certain autoimmune diseases and B cell malignancies warrant simultaneous targeting of multiple disease-relevant signaling pathways. Dual inhibition of spleen tyrosine kinase (SYK) and janus kinase (JAK) represents such a strategy, and may elicit several benefits relative to selective kinase inhibition, such as gaining control over a broader array of disease etiologies, reducing probability of selection for by-pass disease mechanisms, and the potential that an overall lower level suppression of individual targets may be sufficient to modulate disease activity. To this end, we provide data on the discovery and pre-clinical development of PRT062070, an orally active kinase inhibitor which demonstrates activity against SYK and JAK. Cellular assays demonstrated specific inhibitory activity against signaling pathways which utilize SYK and JAK1/3. Limited inhibition of JAK2 was observed, and PRT062070 did not inhibit phorbol 12-myristate 13-acetate (PMA)-mediated signaling or activation in B and T cells, nor T cell antigen receptor-mediated signaling in T cells, providing evidence for selectivity of action. Potent anti-tumor activity was observed in a subset of B cell lymphoma cell lines. Following oral dosing, PRT062070 suppressed inflammation and auto-antibody generation in a rat collagen-induced arthritis model and blocked B cell activation and splenomegaly in a mouse model of chronic B cell antigen receptor stimulation. PRT062070 is currently under evaluation in a phase I dose escalation study in patients with B cell leukemia and lymphoma (NCT01994382), with proof-of-concept studies in humans planned to assess therapeutic potential in autoimmune and malignant diseases.
Introduction

Inhibition of SYK and JAK has demonstrated therapeutic potential in a variety of immunological disorders. SYK is required for signaling and immune cell activation via the B cell antigen receptor (BCR), activating fragment chrystallizable receptors (FcR), and integrins (Mocsai et al., 2010). Genetic studies demonstrate SYK is required for certain inflammatory and autoimmune mechanisms in mice (Colonna et al., 2010; Jakus et al., 2010; Elliott et al., 2011; Wex et al., 2011; Ozaki et al., 2012), a concept which has been reproduced pharmacologically in cellular and animal models (Braselmann et al., 2006; Pine et al., 2007; Coffey et al., 2012). Phase II clinical trials of SYK inhibition by R788 demonstrated efficacy in B cell non-Hodgkin lymphoma (NHL) and chronic lymphocytic leukemia (CLL) (Friedberg et al., 2010), rheumatoid arthritis (RA) (Weinblatt et al., 2008; Weinblatt et al., 2010), and immune thrombocytopenia (Podolanczuk et al., 2009). SYK inhibition by GS-9973 was recently reported to suppress tumor progression in NHL/CLL (Sharman et al., 2013). These data are consistent with the clinical activity observed by inhibition of Bruton’s tyrosine kinase (BTK) (Byrd et al., 2013; Wang et al., 2013) and phosphatidylinositol-4,5-bisphosphate 3-kinase delta (PI3Kδ) (Brown et al., 2013), both downstream of SYK in the BCR signaling pathway. JAK family kinases control immune responses following ligation of interleukin (IL) receptors and a subset of chemokine receptors (Yamaoka et al., 2004). The JAK inhibitor CP690,550 (tofacitinib) is approved for the treatment of RA, and has demonstrated clinical activity in psoriasis (Papp et al., 2012) and the prevention of organ transplant rejection (Vincenti et al., 2012). Moreover, early clinical studies suggest that targeting JAK pathways using small molecule JAK inhibition (Younes et al., 2012), signal transducer and activator of transcription (STAT)3 knock down (Hong et al., 2013), or a neutralizing antibody specific for IL6 (Kurzrock et al., 2013) may be beneficial for the treatment
of B cell malignancies. These data suggest that SYK and JAK independently contribute to the pathogenesis of certain autoimmune and malignant disorders.

Our research has focused on understanding the complexity of cell signaling networks involved in regulating B cell activation and survival. It was previously reported that IL2, IL4, and tumor necrosis factor α act as co-stimulatory molecules to the BCR (Waldmann et al., 1984; Zubler et al., 1984; Clark et al., 1989; Braun et al., 2002), promoting proliferation and survival of healthy normal B cells. We previously reported that co-stimulation of the BCR with IL2 or IL4 in healthy normal B cells significantly enhanced cellular activation relative to BCR or cytokine stimulation alone, and that combining SYK selective and JAK selective inhibitors more potently suppressed this activation response relative to either inhibitor alone (Coffey et al., 2013). Hence, BCR/SYK and cytokine JAK/STAT derived signals appear to act in concert to control normal B cell function. Of particular interest, elevated serum IL6 and IL10 are associated with more aggressive disease progression in CLL and NHL (Fayad et al., 2001; Lai et al., 2002; el-Far et al., 2004). IL4 promotes the survival of CLL cells in culture, and also protects them from death induced by treatment with fludarabine and chlorambucil (Steele et al., 2010). IL6 and IL10 autocrine stimulation promotes the survival of subsets of B cell NHL (Lam et al., 2008).

Combined SYK/JAK inhibition may therefore represent a powerful strategy to control difficult to treat autoimmune diseases and B cell malignancies.

We present herein the discovery and biological characterization of PRT062070, a novel small molecule inhibitor of SYK and JAK family kinases in pre-clinical and clinical development (Flinn et al., 2014) for the treatment of autoimmune disease and B cell malignancies.
Materials and Methods

Synthesis of PRT062070 and purified kinase assays. Extensive structure-activity relationship studies identified the potent and orally bioavailable dual SYK/JAK inhibitor PRT062070, 4-(cyclopropylamino)-2-((4-[4-(ethylsulfonyl)piperazin-1-yl]phenyl)amino)pyrimidine-5-carboxamide hydrochloride. PRT062070 is synthesized via a three step process using starting materials 4-(cyclopropylamino)-2-(methylthio)pyrimidine-5-carboxylic acid and 4-(4-(ethylsulfonyl)piperazine-1-yl)benzenamine. The structure identity and quality of PRT062070 was confirmed by high performance liquid chromatography, fourier transform infrared spectroscopy, high resolution mass spectroscopy, and proton nuclear magnetic resonance. Potency against purified SYK was determined by fluorescence resonance energy transfer as previously described (Coffey et al., 2012). A broader panel of 270 purified kinases (EMD Milipore; Billerica, MA) was surveyed in which PRT062070 was tested at a fixed concentration of 300nM. \[^{33}P\]-labeled substrate was measured after incubation of purified kinase with peptide substrate and \[^{33}P\]ATP at the \(K_m\) concentration for the kinase.

Intracellular signaling in human and rat whole blood. Human whole blood was collected into lithium heparin vacutainers (VWR, Radnor, PA) from the antecubital vein of subjects who gave written informed consent (approved by the Human Subjects Committee of Portola Pharmaceuticals, Inc.). Rat whole blood was collected into lithium heparin via cardiac puncture. 100µl aliquots of whole blood were pre-incubated for 1h at 37°C with various concentrations of PRT062070 (0.1 to 4µM) prior to stimulation. Human whole blood was stimulated for 10min with 2.5µl goat anti-human IgD (Bethyl Laboratories, Montgomery, TX), 10min with 80nM PMA (Sigma-Aldrich, Saint Louis, MO), 10min with 10µl anti-CD3/CD28 Dynabeads (Life Technologies, Grand Island, NY), or 15min with 15ng/ml of \textit{E. coli} derived recombinant human
IL2, IL4, IL6, or GM-CSF (R&D Systems, Minneapolis, MN). Rat whole blood was stimulated for 10min with *E. coli* derived recombinant rat IL6 (R&D Systems). The signaling reaction was terminated by addition of 2ml pre-warmed (37°C) 1x phosflow Lyse/Fix buffer (BD Biosciences, San Jose, CA), followed by incubation at room temperature for 10min. Fixed cells were washed twice in phosphate buffered saline (PBS; Sigma-Aldrich), and re-suspended in -20°C 50% methanol diluted in PBS and stored at 4°C overnight. Unless otherwise indicated, all antibodies used were obtained from BD Biosciences. Following permeabilization of the cell membrane, cells were washed in PBS containing 1% bovine serum albumin (BSA) and re-suspended in the same buffer containing mouse anti-human CD19, rabbit anti-human pERK Y204 (Cell Signaling Technologies, Danvers, MA), and mouse anti-human pSYK Y352 specific antibodies (anti-IgD and PMA stimulations), mouse anti-human CD3, pERK Y204, and mouse anti-human pZap70 Y319 (anti-CD3/CD28 stimulations), mouse anti-human CD3, CD4, CD8, and STAT5 Y694 specific antibodies (human IL2 stimulations), mouse anti-human CD3, CD4, CD8, CD14, CD19, and STAT6 Y641 specific antibodies (human IL4 stimulations); mouse anti-human CD14 and STAT3 Y705 specific antibodies (human IL6 stimulations), mouse anti-human CD14 and STAT5 Y694 specific antibodies (human GM-CSF stimulations), and mouse anti-rat CD3, CD4, CD8, and mouse anti-human and mouse STAT1 Y701 specific antibodies (rat IL6 stimulations) for 1h at room temperature. Cells were then washed and re-suspended in PBS/BSA and assessed by flow cytometry (LSRII; BD Biosciences) for effect of PRT062070 on SYK and JAK dependent signaling.

**Cellular activation in human whole blood and mouse splenocytes.** B cell and basophil activation experiments in human whole blood were performed as previously described (Coffey et al., 2012). In drug combination experiments, PRT062070 potency was compared to that of the
SYK selective (PRT062607/BIIB057; (Coffey et al., 2012)) and JAK selective (CP690,550; tofacitinib) inhibitors, with the SYK and JAK selective inhibitors combined in a 1:1 ratio for direct comparison to PRT062070. IL4-mediated functional responses were performed by stimulating human whole blood overnight with 15ng/ml IL4, followed by staining with mouse antibodies specific for human CD3, CD4, CD8, CD14, CD19, CD23, CD25, and CD69. Mouse splenocytes were obtained from Balb/c mice by forcing the spleens through a 0.45µm single cell strainer (BD Biosciences). Washed cells were stimulated overnight with 1µl goat anti-mouse IgD serum (Ebiosciences, San Diego, CA), or goat serum isotype control (Sigma-Aldrich) in the presence of various concentrations of PRT062070. B cell activation was determined the next day by staining with CD45R/B220, CD69, CD80, and CD86 as described elsewhere (Coffey et al., 2012).

**Tumor cell line viability experiments.** Cell lines obtained from the American Type Culture Collection (Manassas, VA; Ramos RA-1, Daudi, and Toledo) and DSMZ (Braunschweig, Germany; SU-DHL4 and SU-DHL6) were cultured in RPMI media supplemented with 10% fetal bovine serum (0.5x10^6 cells/ml). Cells were treated with the indicated concentrations of PRT062070, the Syk selective inhibitor PRT060318 (Reilly et al., 2010), the pan-JAK inhibitor 1 (EMD Milipore; Darmstadt, Germany), a 1:1 combination of PRT060318 and JAK inhibitor 1, or vehicle control (0.5% DMSO) for 72h. Cell viability assays were performed using CellTiter Glo (Promega; Madison WI) in 384-well plates. Cells were seeded at a density of 5,000 cells per well. Apoptosis was measured using the cleaved caspase 3 detection kit (BD Biosciences) as per the supplied protocol. In some experiments, SU-DHL6 cells were mixed with peripheral blood mononuclear cells isolated from healthy donor whole blood to compare the sensitivity of tumor
versus normal B cells to PRT062070 in the same tube. Normal and tumor B cells were distinguished by FACS based on CD19 expression and side scatter.

**Rat collagen-induced arthritis (CIA) model.** All in vivo studies were completed in compliance with the “Guide for the Care and Use of Laboratory Animals” and with prior approval from Portola’s Institutional Animal Care and Use Committee. The rat CIA model and histopathology evaluation was performed as previously described (Coffey et al., 2012). Briefly, female Lewis rats (7-8 weeks old and 159-187 grams at study initiation) were immunized and boosted with bovine collagen, and then randomized to treatment groups upon development of ankle inflammation scores of 1-2. Treatment was initiated after development of disease symptoms. Rats were then administered vehicle or PRT062070 by oral gavage twice daily for the following two weeks. At study termination, whole blood from the abdominal aorta was collected for complete blood counts and PRT062070 plasma concentration. Blood for pharmacokinetic analysis was collected immediately prior to the final dose (Cnadir), and at 2h and 6h post-final dose for all dose levels. The Tmax for PRT062070 in rats is 2h (data not shown). Serum collagen antibody (IgG) titers were monitored both in a pilot study in naïve, inflammation score 0, inflammation score 1-2, and inflammation score 4-8 rats, as well as at study termination in the PRT062070 efficacy model using the Rat Anti-Type I and Type II Collagen IgG Assay Kit (Chondrex, Redmond, WA).

**Mouse in vivo BCR stimulation.** BCR stimulation leading to cellular activation and splenomegaly was performed as previously described (Spurgeon et al., 2012). Briefly Balb/c mice (n=5 per group) received a single subcutaneous administration of isotype control goat serum or pre-immune mouse IgD goat serum. Oral twice daily administration of vehicle (0.5% methylcellulose in water) or PRT062070 at various dose levels was initiated 1h prior to
inoculation with serum and continued for a total of 5 days. On day 5, mice were anesthetized with SC ketamine cocktail and exsanguinated via cardiac puncture. Six mice were euthanized immediately prior to final dose, and remaining mice (at least 3 per dose group) were euthanized 1h post-final dose, which estimates Tmax in this species (data not shown). At the time of euthanasia, spleens were weighed and single cell suspensions were prepared for evaluation of B cell CD69 and CD80/86 expression by flow cytometry as described above. Plasma was prepared from the blood and analyzed for PRT062070 concentration to estimate drug levels achieved.

**Pharmacokinetic Analysis.** Plasma samples were analyzed for PRT062070 concentration using a liquid chromatography tandem mass spectrometry (LC/MS/MS). In brief, 100 µL plasma samples were processed in a 96-well Captiva™ filter plate (0.2 µm, Varian, Inc., Palo Alto, CA). The samples were precipitated with acetonitrile containing 200 ng/mL of an internal standard. The mixture was vortexed and refrigerated at 4°C for at least 30 minutes to allow for complete protein precipitation. The mixture was filtered into a 96-well collection plate. The filtrate was injected onto a Sciex API3000 LC/MS/MS equipped with a turbo-ion spray source. PRT062070 was separated on a Thermo Hypersil-Keystone C18 column (4.6 x 50 mm, 5 µm; Fisher Scientific, Houston, TX). A mobile phase gradient mixture of 80% mobile phase A (10 mM ammonium acetate in water) and 20% mobile phase B (10 mM ammonium acetate in 10% water 90% acetonitrile) to 75% mobile phase B was programmed over 2.3 minutes. The peak area of the m/z 446→336 product ion (PRT062070) was measured against that of the m/z 357→295 product ion (PRT061196) in positive ion mode. The analytical range was 1 to 10000 ng/mL for PRT062070.

**Statistical Analysis.** Statistical calculations were performed using the R software. The data of the dose response studies were analyzed using the three parameter log-logistic model in the drc
package with the upper limit fixed to 100% inhibition. The confidence intervals of the IC_{50} values were calculated by Taylor expansion. T tests were performed using Excel software, with statistical significance set to P < 0.05.
Results

Characterization of PRT062070 selectivity and potency against SYK and JAK in purified kinase assays. PRT062070 is a reversible ATP-competitive small molecule kinase inhibitor, the chemical structure of which is shown in Fig. 1A. Potency and specificity of PRT062070 was initially tested at 300nM, 10x above the SYK inhibitory IC\textsubscript{50}, in the Millipore panel of 270 independent purified kinase assays. Kinases that were inhibited by >80% were then re-evaluated to determine IC\textsubscript{50} using a 10-point concentration-response curve. Twenty four kinases fit this criterion, and IC\textsubscript{50}'s for each are shown in Fig. 1B, arranged in order of potency of inhibitory activity.

PRT062070 inhibits SYK-dependent signaling and functional responses in B cells. The potency and specificity of SYK inhibition by PRT062070 was tested in human whole blood from normal donors, in which various leukocyte subsets were stimulated via SYK-dependent or SYK-independent pathways. BCR signaling is initiated by activation of the SRC family kinase LYN, which phosphorylates SYK at Y352. SYK then associates with the Igα/β subunits, and propagates the signal to down-stream effectors such as extracellular signal-related kinase (ERK)1/2 (Jiang et al., 1998). Hence, we stimulated B cells in human whole blood (n=7) with antibody specific for the BCR, and measured LYN (SYK Y352) and SYK (ERK Y204) kinase activity. The IC\textsubscript{50} against ERK Y204 phosphorylation was 0.5µM, with no appreciable inhibition of SYK Y352 phosphorylation at 4µM (Fig. 2A). Consistent with the inhibition of SYK, PRT062070 reduced the ability of stimulated B cells to up-regulate cell surface expression of the early activation marker CD69 (IC\textsubscript{50} = 0.11µM). Inhibition of FcεRI mediated basophil degranulation (IC\textsubscript{50} = 0.12µM) further confirmed the potency of SYK inhibition in whole blood (Fig. 2B). PRT062070 did not inhibit PMA-induced protein kinase C-mediated signaling to
ERK Y204, nor PMA induced CD69 up-regulation in B cells at 4µM (Fig. 2C). Further, T cell signaling following cross-linking of the T cell antigen receptor or in response to PMA was unaffected by 4µM PRT062070 (Fig. 2D), demonstrating specificity of action and lack of broad cross-reactivity to other pathways.

**Inhibition of cytokine signaling and IL4 functional responses in human whole blood.**

Cytokine stimulations were performed in human whole blood to assess the potency of PRT062070 against JAK-STAT signaling pathways. IL2 (JAK1/3) stimulation resulted in STAT5 Y694 phosphorylation in T cells. The PRT062070 IC₅₀’s were 0.3µM and 0.16µM in CD4+ and CD8+ T cells, respectively (Fig. 3A). IL4 (JAK1/3) stimulation resulted in phosphorylation of STAT6 Y641 in CD4+ T cells, CD8+ T cells, CD14+ monocytes, and CD19+ B cells; PRT062070 demonstrated IC₅₀’s of 0.58µM, 0.33µM, 1µM, and 0.92µM, respectively (Fig. 3B). IL6 (JAK1) stimulation leading to STAT3 Y705 phosphorylation in monocytes was inhibited with an IC₅₀ of 0.35µM (Fig. 3C), whereas GM-CSF (JAK2) induced STAT5 Y694 phosphorylation in monocytes was not potently inhibited by this compound (Fig. 3D), indicating an enhanced inhibitory potency of PRT062070 against signaling pathways that utilize JAK1/JAK3 relative to JAK2 in cellular systems. IL4 stimulation results in the up-regulation of various cell surface activation markers in leukocytes. We therefore incubated human whole blood with IL4 overnight in the presence of various concentrations of PRT062070 and measured the impact on functional responses in cells. In the presence of IL4, B cells up-regulate cell surface CD23, CD25, and CD69; the PRT062070 IC₅₀ for each were 1.5µM, 0.26µM and 0.13µM, respectively (Supplemental Figure 1, left panel). Monocytes up-regulate cell surface CD23 and CD25 expression, which were inhibited by PRT062070 with IC₅₀’s of 0.23µM and 0.36µM, respectively (Supplemental Figure 1, right panel).
BCR-induced cellular activation is potentiated by cytokine co-stimulation (Waldmann et al., 1984; Zubler et al., 1984; Clark et al., 1989; Braun et al., 2002; Coffey et al., 2013). Consistently, BCR-induced activation was more sensitive to the effects of PRT062070 (CD69 IC$_{50}$ = 0.11µM; Fig. 2A) than would have been predicted from the potency of cellular SYK kinase inhibition following the same stimulation (ERK Y204 IC$_{50}$ = 0.5µM; Fig. 2A). This difference in potency between inhibition of BCR-mediated SYK signaling and B cell activation was not observed with SYK selective inhibition, where overlapping IC$_{50}$’s were reported in these two assays (Coffey et al., 2012). To corroborate this observation, we tested the ability of selective SYK versus JAK inhibition alone and in combination, relative to PRT062070, to suppress BCR-mediated CD69 up-regulation in human whole blood. SYK inhibition alone (PRT062607/BIIB057) resulted in full suppression of CD69 up-regulation with an IC$_{50}$ of approximately 0.3µM. Interestingly, whereas JAK inhibition had only a partial effect in suppressing B cell function, the combination of SYK and JAK selective inhibitors exerted greater suppression than did either inhibitor alone, which mimicked the potency of PRT062070 in this assay (Supplemental Figure 2). These data indicate that SYK and JAK contribute to the overall response of B cells to BCR ligation, and provide evidence that PRT062070 affects BCR-mediated B cell activation by dual inhibition of SYK and JAK.

**SYK and JAK cooperate to reduce viability in a subset of NHL cell lines.** Cell lines with differential sensitivities to SYK and JAK selective inhibition were tested for survival in the presence of combined SYK plus JAK selective inhibitors and the dual SYK/JAK inhibitor PRT062070. As shown in Fig. 4A, the germinal center subtype (GCB) diffuse large B cell lymphoma (DLBCL) cell lines SU-DHL4 and SU-DHL6 were sensitive to SYK selective (PRT060318; top left panel) but not to pan-JAK (JAK Inhibitor 1; top right panel) inhibition.
This contrasts with two Burkitt lymphoma cell lines, Ramos and Daudi, which demonstrated comparable sensitivity to both SYK and JAK kinase inhibition. Combining the SYK and JAK selective inhibitors (Fig. 4A, bottom left panel) resulted in greater inhibition of cell viability relative to either inhibitor alone. This conclusion was supported by the IC\textsubscript{50}’s with non-overlapping 95% confidence intervals (Fig. 4B). The 95% confidence interval for inhibition of Ramos cell viability was 0.49-0.72\(\mu\)M with combined SYK plus JAK selective inhibition, versus 0.77-1.77\(\mu\)M and 0.98-2\(\mu\)M for SYK and JAK selective inhibition, respectively. Similarly, the 95% confidence interval for inhibition of Daudi cell viability was 0.85-1.15\(\mu\)M with combined SYK plus JAK selective inhibition, versus 1.77-3.54\(\mu\)M and 1.21-2.85\(\mu\)M for SYK and JAK selective inhibition, respectively. PRT062070 (Fig. 4A; bottom right panel) resulted in viability effects similar to that of the combined SYK plus JAK selective inhibition, although the confidence intervals did overlap with that of SYK and JAK selective inhibitors (Fig. 4B).

Additional studies were conducted to evaluate the potential for broad cytotoxic effects on B cells. For these experiments, SU-DHL4, SU-DHL6, and Ramos cells were used, each of which express functional cell surface BCR’s. By comparison, the Toledo cell line of DLBCL origin does not express surface BCR, and has thus adapted BCR-independent survival mechanisms (Gabay et al., 1999). As shown in Fig. 5A, SU-DHL4, SU-DHL6, and Ramos cells each underwent apoptosis in response to treatment with PRT062070, whereas there was no detectable activity in the BCR-negative Toledo cell line. SU-DHL6 tumor cells were added to healthy normal human peripheral blood mononuclear cells, and treated with PRT062070 to determine the relative sensitivity of tumor versus non-tumor B cells. As shown in Fig. 5B, nearly 80% of the tumor B cells underwent apoptosis in the presence of PRT062070, while under the same conditions there was no effect on the non-tumor primary B cells.
**PRT062070 elicits dose-dependent efficacy in the rat CIA model.** We tested the potential for PRT062070 to modulate inflammation in the rat CIA treatment model following oral dosing. Animals treated with vehicle control exhibited a rapid onset of hind paw inflammation within 2-3 days of boosting with adjuvant, with maximal inflammation occurring by day seven. Treatment with 0.5mg/kg PRT062070 (attaining average Cmax plasma concentration at 2h of 0.18µM) resulted in a non-statistically significant trend toward reduced ankle inflammation, while significant reductions in inflammation were achieved with the 1.5, 3, and 5mg/kg doses, with average Cmax plasma concentrations at 2h of 0.52µM, 0.58µM, and 1.49µM, respectively. Inflammation was abolished at the 3mg/kg dose, and reversed relative to pre-treatment levels at 5mg/kg (Fig. 6A). Blinded histological observations were consistent with the inflammation scores. Significant improvements in inflammatory infiltrate within the synovium and the integrity of the articular cartilage were observed in a dose-dependent manner (Fig. 6B). Representative histological evaluations are shown in Supplemental Figure 3.

As expected, PRT062070 treatment also affected anti collagen antibody formation. Relative to the time of treatment initiation (ankle inflammation score of 1-2), circulating levels of anti-collagen antibody approximately doubled in control animals with inflammation scores of 4-8 over the experimental time-course of 16 days (Fig. 6C). In animals treated with PRT062070, we observed a 50% reduction in circulating antibody titers at the 1.5, 3, and 5mg/kg doses (Fig. 6D). These data suggest that a threshold plasma concentration of PRT062070 was reached at which continued terminal differentiation of mature B cells to antibody-secreting plasma cells was disrupted. Importantly, the anti-inflammatory effects of PRT062070 occurred within the pharmacologically active range of this compound (i.e. 0.52-1.49µM). To estimate the level of target inhibition achieved by these concentrations in rat blood, we performed IL6 stimulations in
the presence of a range of PRT062070 concentrations. The IC$_{50}$ for IL6 signaling in CD3+ T
cells was 1.4µM (Supplemental Figure 4). Relating this to average Cmax plasma concentrations
observed in the rat CIA model, 0.52uM to 1.49uM achieved approximately IC$_{25}$ to IC$_{50}$ against
this pathway. These data suggest that a low level of combined SYK and JAK inhibition is likely
sufficient for meaningful anti-inflammatory effects in this stringent model.

**Inhibition of chronic BCR stimulation and splenomegaly in mice.** Chronic BCR stimulation
in secondary lymphoid organs may contribute to the proliferation and survival of certain B cell
malignancies. Chronic BCR signaling in vivo can be modeled using subcutaneous
administration of an activating anti-mouse BCR antibody, which was previously shown to induce
immune cell activation in vivo (Mountz et al., 1987), BCR signaling in mouse whole blood
(Coffey et al., 2012), and a rapid onset of splenomegaly in mice (Spurgeon et al., 2012).
PRT062070 inhibited mouse splenic B cell activation in response to this antibody in vitro (Fig.
7A) and in vivo (Fig. 7B). Fig. 7B depicts spleens from 2 mice representative of each treatment
group stimulated with isotype control goat serum and treated with vehicle (first set of spleens),
stimulated with anti-BCR and treated with vehicle (second set of spleens), and stimulated with
anti-BCR but treated with 15mg/kg PRT062070 (third set of spleens). As shown in the bar graph
of Fig. 7B, PRT062070 also suppressed up-regulation of splenic B cell surface CD80/86 and
CD69 by > 60%. The inhibition of splenomegaly was dose and concentration-dependent (Figs.
7C and 7D). Average Cmax plasma concentrations were 15, 213, 1210, 1395, and 1913ng/ml
for the 1, 5, 15, 20, and 30mg/kg dose groups, respectively. Statistically significant effects on
splenomegaly relative to control were achieved at Cmax concentrations of 1210ng/ml and
greater. These data demonstrate that PRT062070 can inhibit BCR signaling and activation in the
spleen following oral dosing in mice.
Discussion

We describe herein the pre-clinical characterization of the kinase inhibitor, PRT062070. In the Millipore Kinase Profiler screen of 270 purified kinases, PRT062070 inhibited twenty four kinases by more than 80% when tested at a concentration ten-fold above SYK IC₅₀. The purified kinase screen was used to help initially define the kinase-inhibitory profile of PRT062070, enabling a more focused evaluation in cellular assays. Potency in the purified kinase assays largely predicted potency in cellular assays. The major exception was lack of cellular potency against JAK2, providing evidence for potential false-positives in the purified kinase assays. Moreover, the rank-order of potency for SYK and JAK family members in the purified kinase assays did not translate directly into rank-order in cellular assays, likely reflecting the greater complexity of signaling pathways within cells at physiological ATP concentration.

We observed very comparable potency in cellular assays measuring JAK/STAT, BCR/SYK, and FcεRI/SYK, with limited to no activity against signaling pathways utilizing PKC, LCK, and ZAP70. Consistent with this kinase-inhibitory profile, PRT062070 in a dose-dependent manner suppressed inflammation and auto-antibody generation in the rat CIA model, and prevented BCR-mediated splenomegaly and B cell activation in mice.

Small molecule inhibitors of SYK and JAK have independently demonstrated activity in rodent inflammation models (Fridman et al., 2010; Coffey et al., 2012). B cell activation is required for disease development in the rat CIA and mouse BCR-induced splenomegaly models. We therefore studied the relative contributions of SYK and JAK to B cell activation in human whole blood, enabling more control over the pharmacological mechanisms. We found that by combining SYK and JAK inhibitors we could not only more potently inhibit B cell functional responses in human whole blood, but that this particular combination mimicked the potency
observed with PRT062070, supporting a dual SYK/JAK mechanism for B cell inhibition by this molecule. This data is consistent with the observation that combined SYK and JAK inhibitors mimicked the potency of PRT062070 in Burkitt lymphoma cell lines. It is therefore likely that dual SYK/JAK inhibition is at least partly responsible for the activity seen with PRT062070 in the various ex vivo and in vivo models presented here. We cannot, however, rule out the possibility that inhibition of other kinases contribute to the over-all activity of PRT062070.

The same cell signaling networks that promote inflammation and autoimmunity also appear to promote the survival and proliferation of certain B cell malignancies. In particular, SYK is an intriguing target for CLL, which appears to be heavily dependent upon signaling via the BCR (Chiorazzi et al., 2005; Duhren-von Minden et al., 2012). This is evidenced by the impressive clinical responses observed with SYK (fostamatinib; (Friedberg et al., 2010), BTK (ibrutinib; (Byrd et al., 2013) and PI3Kδ (idelalisib; (GS1101; CAL-101) (Brown et al., 2013)) inhibition. SYK is upstream of BTK and PI3Kδ on the BCR pathway (Jiang et al., 1998; Beitz et al., 1999; Hatton et al., 2012; Sharman et al., 2013), which allows us to postulate that targeting this kinase may more broadly affect BCR signaling. SYK is also required for integrin signaling, and integrin-mediated adhesion of CLL to stromal cells, facilitating maintenance and survival in the tumor microenvironment (Mocsai et al., 2002; Frommhold et al., 2007; Buchner et al., 2010). Hence, inhibition of SYK alone is likely to affect multiple disease-relevant survival mechanisms in vivo. In addition, serum levels of several cytokines are increased in CLL (Mahadevan et al., 2009), and have predictive value in disease outcome (Fayad et al., 2001; Lai et al., 2002; Yan et al., 2011). IL2, IL4, and alpha interferon promote CLL survival in vitro, reportedly by inducing the up-regulation of B cell lymphoma 2 family proteins (Dancescu et al., 1992; Panayiotidis et al., 1993; Jewell et al., 1994; Castejon et al., 1999). Stimulation of CLL cells with IL4 induces
up-regulation of B cell lymphoma-XL and myeloid leukemia cell differentiation protein, and protects CLL from apoptosis via cytotoxic agents (Steele et al., 2010). Thus JAK inhibition may provide therapeutic benefit in CLL patients, particularly in combination with SYK inhibition.

Subsets of NHL also demonstrate a reliance on SYK and JAK pathways for survival. DLBCL has been discriminated genetically into several subtypes, most notably the GCB and activated B cell type (ABC) (Alizadeh et al., 2000). The ABC/GCB designation appears to predict survival (Rosenwald et al., 2002; Wright et al., 2003) as well as response of tumor cell lines to small molecule kinase inhibition of the BCR signaling pathway (Davis et al., 2010). Based on work using cell lines and primary tumors, the clinical response to BTK inhibition (ibrutinib) in DLBCL was genetically defined, and appears to be determined by the lymphoma subtype (GCB or ABC) and by the occurrence of mutations that drive nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) mediated JAK/pSTAT3 activation (Davis et al., 2010). BTK inhibition did not yield clinical responses in patients with GCB subtype DLBCL (overall-response rate was 5%) (Wilson et al., 2012); it should be noted that this subtype represents approximately 50% of all DLBCL cases. No responses were seen in patients with myeloid differentiation primary response gene 88 (MYD88) mutation alone (Wilson et al., 2012), and experimental evidence suggests that no responses may be seen in patients with caspase recruitment domain-containing protein 11 (CARD11) mutations (Davis et al., 2010). In a separate study, 43% of DLBCL could be characterized by amplification of the BCR pathway (termed BCR/Proliferation), and interestingly, 25% were characterized by the infiltration of non-tumor leukocytes to the tumor environment (termed Host Response) (Monti et al., 2005). In the host response cluster, there was evidence for gamma interferon signaling and increased expression of receptors for IL2, IL6, and IL15. Targeting SYK and JAK may therefore be
relevant to not only tumor-intrinsic pro-survival signaling, but also to suppressing pro-survival signals provided by the tumor microenvironment.

Of interest, SYK inhibition appears to affect a survival mechanism in GCB subtype lymphoma cell lines that BTK inhibition does not affect (Figs. 4-5 and (Cheng et al., 2011)), and therefore SYK inhibition may have distinct clinical activity in this population. Moreover, subsets of ABC subtype NHL cell lines secrete IL6 and/or IL10, engaging an autocrine JAK/pSTAT3 signaling pathway which contributes to survival (Ding et al., 2008; Lam et al., 2008). Dual targeting of these survival signals via IKKB and JAK inhibition more effectively killed ABC DLBCL cell lines relative to either inhibitor alone (Lam et al., 2008), suggesting that dual inhibition of BCR and JAK/STAT signaling pathways may have an advantage over selective inhibition of the BCR pathway alone. This hypothesis was recently tested genetically; combined short hairpin RNA knockdown of SYK and JAK was found to have greater anti-tumor activity in a DLBCL cell line relative to knock down of each kinase alone (Ma et al., 2013). Consistently, broad anti-tumor activity in DLBCL cell lines was observed with PRT062070, including cell lines bearing MYD88 and CARD11 mutation (Ma et al., 2013). Our data are consistent with previously published reports where DLBCL cell lines of GCB origin demonstrated no sensitivity to JAK inhibition (Ding et al., 2008; Lam et al., 2008). By comparison two Burkitt lymphoma cell lines demonstrated sensitivity to SYK and JAK inhibition separately, and enhanced sensitivity when combined (Fig. 4). We are currently conducting studies to evaluate the dependency of a much broader panel of genetically diverse DLBCL cell lines on SYK and JAK survival pathways. This will include a pharmacogenomic evaluation to identify mutations that influence the cells dependencies on SYK and JAK pathways for survival. Ongoing pharmacodynamic evaluation in
B cell lymphoma and leukemia patients treated with PRT062070 (Flinn et al., 2014) should further elucidate the specific contribution of SYK and JAK pathways to disease pathology.
Acknowledgements

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Authorship Contributions

Participated in research design: Coffey, Betz, Pak, Baker, Hollenbach, Pandey, Sinha
Conducted experiments: Coffey, DeGuzman, Inagaki, Baker
Contributed new reagents or analytic tools: Pandey
Performed data analysis: Coffey, Betz, DeGuzman
Wrote or contributed to the writing of the manuscript: Coffey, Betz, Baker, Hollenbach, Pandey, Sinha
References


Footnotes

a) This work was supported by Portola Pharmaceuticals, Inc.

b) Reprint requests can be made to:

Greg Coffey
Portola Pharmaceuticals
270 E. Grand Ave
South San Francisco, CA 94080
Phone: (650)246-7565
Email: gcoffey@portola.com
Figure Legends

Fig. 1: PRT062070 chemical structure and potency in purified kinase assays.  A) The chemical structure of PRT062070 is shown.  B) PRT062070 was screened against the Millipore panel of 270 purified kinases in duplicate at 10-fold above its SYK IC$_{50}$.  Kinases inhibited by greater than 80% were then tested against a 10-point PRT062070 concentration-response curve to determine IC$_{50}$.  The IC$_{50}$’s for each of these kinases are shown in order of potency.

Fig. 2: Inhibition of SYK-mediated signaling and functional responses in human whole blood.  For each graph, the mean percent inhibition ± SEM of the mean fluorescent intensity (MFI) normalized to vehicle control is reported on the y-axis and PRT062070 concentration (µM) is reported on the x-axis.  A) Human whole blood was stimulated with anti-IgD to measure LYN kinase activity (pSYK Y352; open triangle, n=7), SYK kinase activity (pERK Y204; open circle, n=8), and B cell functional response (CD69 up-regulation; closed triangle, n=17).  B) FcεRI mediated basophil degranulation in human whole blood (n=4).  C) Human whole blood stimulated with PMA (n=6) to induce PKC-dependent pERK Y204 (closed circles) and CD69 up-regulation (open circles) on B cells.  D) Human whole blood stimulated with anti-CD3/28 dynabeads or PMA (n=6) to induce Zap70- or PKC-dependent, respectively, pERK Y204 (closed circles) and pZap70 Y319 (open circles) in T cells.  Horizontal bars in C and D depict the median.

Fig. 3: PRT062070 exhibits differential potency against cytokine JAK/STAT signaling pathways.  Eight-point concentration response curves (n=6-8) were performed in human whole blood stimulated with IL2 (A), IL4 (B), IL6 (C), or GM-CSF (D).  Percent inhibition of STAT phosphorylation is depicted on the y-axis (mean percent inhibition ± SEM of MFI).  Cell types
affected include CD4- and CD8-positive T cells, CD14-positive monocytes, and CD19-positive B cells. The x-axis shows the concentrations of PRT062070 (µM) tested.

**Fig. 4: SYK and JAK contribute to the survival of a subset of NHL cell lines.** A) concentration-response curves are shown in the presence of the selective SYK inhibitor PRT060318 (top left panel), the pan-JAK inhibitor 1 (top right panel), combined SYK plus JAK selective inhibitors (bottom left panel), and the dual SYK/JAK inhibitor PRT062070 (bottom right panel). Concentration in µM is displayed on the x-axis and percent inhibition of cell viability is shown on the y-axis. Data represent the mean ± SEM of three independent experiments. B) The table depicts the IC₅₀ and 95% confidence intervals for each treatment condition.

**Fig. 5: PRT062070 induces apoptosis in BCR-signaling competent NHL cell lines.** A) The percent of cells expressing cleaved caspase 3 following 48h treatment with vehicle (Vh), 1µM or 3µM PRT062070 is plotted on the y-axis (mean + SD; n=3). B) The scatter plots depict side scatter (SSC; y-axis) and CD19 expression (x-axis) for a mixture of SU-DHL6 cells and healthy human volunteer peripheral blood mononuclear cells. In this manner, SU-DHL6 and primary B cells were resolved from each other and separately gated, as indicated by the arrows. Histograms depict extent of caspase 3 cleavage in primary B cells (top row) and SU-DHL6 (bottom row) following 48h treatment with vehicle or 3µM PRT062070.

**Fig. 6: Dose responsive effect of PRT062070 in rat CIA treatment model.** A) Hind paw inflammation scores (mean ± SEM; y-axis; n=8 per group) are plotted over time in days (x-axis) during the course of treatment with vehicle (open circle), 0.5mg/kg (closed circle), 1.5mg/kg (closed triangle), 3mg/kg (open triangle pointing up), and 5mg/kg PRT062070 (open triangle.
pointing down). The average Cmax plasma concentrations (2h post final dose) achieved in each dosing group is shown at the right of the graph in µM. B) Corresponding mean modified Mankin histopathology scores (± SEM; y-axis) determined in a blinded fashion are presented, with the treatment conditions shown on the x-axis. C and D) Rat serum anti-collagen antibody titers (µg/ml; y-axis) in which titers from naïve, immunized but non-inflamed (score 0), and inflamed rats at scores of 1-2 and 4-8 are shown from a pilot study (C), and titers from naïve and immunized rats treated with vehicle or various PRT062070 dose levels (mg/kg; x-axis) from a treatment study are shown (D). Horizontal bars depict the median. Statistically significant differences relative to vehicle control treated rats are identified with asterisks (P < 0.05).

**Fig. 7: PRT062070 blocks BCR-induced B cell activation and splenomegaly in mice.**

A) FACS plots represent mouse splenic B cells stimulated with isotype control (control) or anti-BCR antibody in the presence of vehicle or PRT062070 at 0.25µM and 0.05µM. B-cell activation (mean + SD, n=4) was measured by up-regulation of CD69 (bottom row) and CD80/86 (top row). B) Mice (n=5 per group) were injected subcutaneously with control goat serum or serum pre-immunized with mouse IgD (anti-BCR). The photograph depicts two representative spleens from each group of mice treated with control goat serum and vehicle control (first set; no stimulation), anti-BCR with vehicle (second set; induced B cell activation), and anti-BCR with 15mg/kg PRT062070 (third set; B cell activation inhibited by PRT062070). The bar graph beneath the picture (mean + SD, n=5) depicts CD69 and CD80/86 surface expression levels on splenic B cells isolated from the anti-BCR administered animals treated with vehicle or 15mg/kg PRT062070. C) Percent inhibition of splenomegaly (determined by normalization to mice treated with anti-BCR and vehicle) is plotted as a bar graph (n = 4-5 mice.
per group; mean ± SD), with the PRT062070 dose levels depicted on the x-axis. D) 
Cmax plasma concentrations of PRT062070 (ng/ml; x-axis) is plotted against the percent inhibition of splenomegaly observed in each individual animal on study (y-axis). Statistically significant differences relative to vehicle control treated mice are identified with asterisks (P < 0.05).
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Fig. 2

A) B cell Response to anti-BCR

B) Basophil Response to anti-FceRI

C) B cell Response to PMA

D) T cell Response to CD3/28 or PMA
Fig. 4

A) 

B) 

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Fig. 5

A) Bar graph showing the percentage of Caspase 3 positive cells (Mean ± S.D.) for different cell lines: SU-DHL4, SU-DHL6, Ramos, and Toledo. The bars represent different treatments: Vh, 1µM PRT062070, and 3µM PRT062070.

B) Flow cytometry analysis comparing the SSC (Side Scatter) and CD19 PerCP (Peridinin-Chlorophyll-Protein) channels for Vehicle and 3µM PRT062070 treatments. The graphs illustrate the percentage of primary B cells and SU-DHL6 cells.
Fig. 6

A) 

Inflammation Score (mean ± SEM)

B) 

Histopathology Score (mean + SEM)

C) 

$10^3$ Collagen Ab Titer (µg/ml)

D) 

$10^3$ Collagen Ab Titer (µg/ml)

[Graphs and data points are shown, representing various treatments and their effects on inflammation and collagen antibody titers.]

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Fig. S1: Inhibition of IL4-induced cellular activation in human whole blood by PRT062070. Whole blood from healthy donors (n=4) was stimulated overnight with IL4 to induce cellular activation in the presence of various concentrations of PRT062070, as shown on the x-axis. Percent inhibition of the various activation markers (CD69, CD25, and CD23) are presented on the y-axis. Activation responses in B cells are shown in the left panel, and responses in monocytes are shown in the right panel. The IC50 for each measure of cellular activation is presented on the graph.
Journal of Pharmacology and Experimental Therapeutics

The dual SYK/JAK inhibitor PRT062070 (Cerdulatinib) demonstrates efficacy in models of autoimmunity and B cell cancer

Greg Coffey, Andreas Betz, Francis DeGuzman, Yvonne Pak, Mayuko Inagaki, Dale C Baker, Stanley J Hollenbach, Anjali Pandey, and Uma Sinha

Fig. S2: PRT062070 potency against BCR-mediated B cell function is mimicked by combined SYK and JAK selective inhibition. BCR-induced CD69 up-regulation was evaluated in human whole blood in the presence of SYK selective (PRT062607), JAK selective (CP690,550), combined SYK and JAK selective (PRT062607 + CP690,550) and dual SYK/JAK (PRT062070) inhibitors. CD69 MFI normalized to percent of vehicle control is plotted on the y-axis (mean ± SEM). The asterisks represent significant differences when compared to selective inhibition of SYK or JAK alone (P < 0.05).
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Fig. S3: PRT062070 protects rats from tissue damage in the CIA model. Panels A-F are H&E stained (A,B,D, and E) or Safranin O stained (C and F) sections of tissue from rats immunized with collagen and subsequently orally treated with either vehicle (A-C) or 5mg/kg PRT062070. A) There is cartilage thinning (arrow) and loss of cartilage (arrowhead). B) The joints also had infiltrates of neutrophils and macrophages tangled in fibrin (arrow) in the stroma surrounding the joint (red asterisks) and free in the lumen (arrow). C) The surface hyaline cartilage had decreased affinity for safranin O (C, white asterisks) with cartilage only staining with the blue counter-stain rather than red as in normal cartilage (F, white asterisks). D) Normal appearing joint structure in rat dosed 5mg/kg PRT062070. E) Cartilage is normal in rat dosed 5mg/kg PRT062070, and stains normally with Safranin O (F).
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Fig. S4: Inhibition of IL6 signaling by PRT062070 in rat whole blood. Concentration-response curve following stimulation of rat whole blood with IL6 (n=4) in the presence of the indicated concentrations of PRT062070 (x-axis). Percent inhibition of STAT1 Y701 phosphorylation in T cells is depicted on the y-axis. The observed IC\textsubscript{50} of 1.4µM is shown on the graph.