The Novel Kinase Inhibitor PRT062070 (Cerdulatinib) Demonstrates Efficacy in Models of Autoimmunity and B-Cell Cancer

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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 bypass 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 preclinical development of PRT062070 [4-(cyclopentyloxycarbonyl)-2-[[4-[4-(ethylsulfonyl)piperazin-1-yl]phenyl]amino]pyrimidine-5-carboxamide hydrochloride], an orally active kinase inhibitor that demonstrates inhibitory activity against signaling pathways that use SYK and JAK1/3. Limited inhibition of JAK2 was observed, and PRT062070 did not inhibit phorbol 12-myristate 13-acetate-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 antitumor activity was observed in a subset of B-cell lymphoma cell lines. After oral dosing, PRT062070 suppressed inflammation and autoantibody 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 spleen tyrosine kinase (SYK) and Janus kinase (JAK) demonstrated therapeutic potential in a variety of immunologic disorders. SYK is required for signaling and immune cell activation via the B-cell antigen receptor (BCR), activating fragment crystallizable receptors, and integrins (Mocsai et al., 2010). Genetic studies demonstrate SYK is required for certain inflammatory and autoimmune mechanisms in mice (Coloma et al., 2010; Jakus et al., 2010; Elliott et al., 2011; Wex et al., 2011; Ozaki et al., 2012), a concept that 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 [6-(5-fluoro-2-(3,4,5-trimethoxyphenylamino)pyrimidin-4-ylamino)-2,2-dimethyl-3-oxo-2,3-dihydropyrido[3,2-b][1,4]oxazin-4-ylmethyl dihydrogen phosphate] 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, 2010), and immune thrombocytopenia (Podolanczuk et al., 2009). SYK inhibition by GS-9973 [6-(1H-indazol-6-yl)-N-(4-morpholinophenyl)imidazo[1,2-a]pyrazin-8-amine] 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 δ (PI3Kδ) (Brown et al., 2013), both downstream of SYK in the BCR signaling pathway. JAK family kinases control immune responses after ligation of interleukin (IL) receptors and a subset of chemokine receptors (Yamaoka et al., 2004). The JAK inhibitor CP690,550 [tofacitinib; (3R,4R)-4-methyl-3-(methyl-7H-pyrrrolo[2,3-d]pyrimidin-4-ylamino)-β-oxo-1-piperidinepropanenitrile] is approved for the treatment of RA and has demonstrated clinical activity in psoriasis (Papp et al., 2013). Inhibition of spleen tyrosine kinase (SYK) and Janus kinase (JAK) demonstrated therapeutic potential in a variety of immunologic disorders. SYK is required for signaling and immune cell activation via the B-cell antigen receptor (BCR), activating fragment crystallizable receptors, and integrins (Mocsai et al., 2010). Genetic studies demonstrate SYK is required for certain inflammatory and autoimmune mechanisms in mice (Coloma et al., 2010; Jakus et al., 2010; Elliott et al., 2011; Wex et al., 2011; Ozaki et al., 2012), a concept that 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 [6-(5-fluoro-2-(3,4,5-trimethoxyphenylamino)pyrimidin-4-ylamino)-2,2-dimethyl-3-oxo-2,3-dihydropyrido[3,2-b][1,4]oxazin-4-ylmethyl dihydrogen phosphate] 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, 2010), and immune thrombocytopenia (Podolanczuk et al., 2009). SYK inhibition by GS-9973 [6-(1H-indazol-6-yl)-N-(4-morpholinophenyl)imidazo[1,2-a]pyrazin-8-amine] 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 δ (PI3Kδ) (Brown et al., 2013), both downstream of SYK in the BCR signaling pathway. JAK family kinases control immune responses after ligation of interleukin (IL) receptors and a subset of chemokine receptors (Yamaoka et al., 2004). The JAK inhibitor CP690,550 [tofacitinib; (3R,4R)-4-methyl-3-(methyl-7H-pyrrrolo[2,3-d]pyrimidin-4-ylamino)-β-oxo-1-piperidinepropanenitrile] is approved for the treatment of RA and has demonstrated clinical activity in psoriasis (Papp et al., 2013).
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 knockdown (Hong et al., 2013), or a neutralizing antibody specific for IL-6 (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 IL-2, IL-4, and tumor necrosis factor α act as costimulatory molecules to the BCR (Waldmann et al., 1984; Zuber et al., 1984; Clark et al., 1989; Braun et al., 2002), promoting proliferation and survival of healthy normal B cells. We previously reported that costimulation of the BCR with IL-2 or IL-4 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 IL-6 and IL-10 are associated with more aggressive disease progression in CLL and NHL (Fayad et al., 2001; Lai et al., 2002; el-Far et al., 2004). IL-4 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). IL-6 and IL-10 autocrine stimulation promote 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 biologic characterization of PRT062070 [4-(cyclopentylamino)-2-[(4-[4-(ethyloxy)alkyl)piperazin-1-yl]phenyl]amino)pyrimidine-5-carboxamide hydrochloride], a novel small molecule inhibitor of SYK and JAK family kinases in preclinical 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. PRT062070 is synthesized via a three-step process using starting materials 4-cyclopentylpropylamine-2-(4-[4-(ethyloxy)alkyl)piperazin-1-yl]benzeneamine. The structure and quality of PRT062070 was confirmed by high-performance liquid chromatography, Fourier transform infrared spectroscopy, high-resolution mass spectroscopy, and proton NMR. 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 300 nM. [32P]-labeled substrate was measured after incubation of purified kinase with peptide substrate and [32P]ATP at the Km concentration for the kinase.

Intracellular Signaling in Human and Rat Whole Blood. Human whole blood was collected into lithium heparin via cardiac puncture. Aliquots (100 μl) of whole blood were preincubated for 1 hour at 37°C with various concentrations of PRT062070 (0.1 to 4 μM) before stimulation. Human whole blood was stimulated for 10 minutes with 2.5 μg anti-human IgD (Bethyl Laboratories, Montgomery, TX), 10 minutes with 80 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO), 10 minutes with 10 μl anti-CD3/CD28 Dynabeads (Life Technologies, Grand Island, NY), or 15 minutes with 15 ng/ml of Escherichia coli-derived recombinant human IL-2, IL-4, IL-6, or granulocyte-macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN). Rat whole blood was stimulated for 10 minutes with E. coli-derived recombinant rat IL-6 (R&D Systems). The signaling reaction was terminated by addition of 2 ml prewarmed (37°C) 1× phosphaef lys/fix buffer (BD Biosciences, San Jose, CA) followed by incubation at room temperature for 10 minutes. Fixed cells were washed twice in phosphate-buffered saline (PBS; Sigma-Aldrich) and resuspended 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. After permeabilization of the cell membrane, cells were washed in PBS containing 1% bovine serum albumin and resuspended in the same buffer containing mouse anti-human CD19, rabbit anti-human p-extracellular signal-regulated kinase (ERK) Y204 (Cell Signaling Technologies, Danvers, MA), and mouse anti-human pSYK Y352 (anti-p-Idg 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, CD69, and STAT5 Y694 (anti-CD3/CD28 stimulations); mouse anti-human CD3, CD4, CD8, and CD69. The phosphorylation of human pSTAT3 Y705 (anti-IgM and PMA stimulations); mouse anti-human CD14 and STAT3 Y705 (anti-IgM stimulations), mouse anti-human CD14 and STAT5 Y694 (anti-IgM stimulations); mouse anti-human CD14 and STAT3 Y705 (anti-IgM stimulations); mouse anti-human CD14 and STAT5 Y694 (anti-IgM stimulations); and mouse anti-human CD14 and STAT5 Y705 (anti-IgM stimulations) for 1 hour at room temperature. Cells were then washed and resuspended in PBS/bovine serum albumin 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 with 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. Human whole blood were performed as previously described (Coffey et al., 2012). In drug combination experiments, PRT062070 potency was compared with 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 with the SYK-selective [PRT060318, JAK inhibitor 1 (EMD Milipore, Darmstadt, Germany), a 1:1 combination of PRT060318 and JAK inhibitor 1, or vehicle control (0.5% dimethylsulfoxide)].
for 72 hours. Cell viability assays were performed using CellTiter Glo (Promega, Madison WI) in 384-well plates. Cells were seeded at a density of 5000 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 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 collagen-induced arthritis (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 g 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 5 days. On day 5, mice were injected into a Sciex API3000 liquid chromatography–tandem mass spectrometry equipped with a turbo-ion spray source. PRT062070 was separated on a Thermo Hyperil-Keystone C18 column (4.6 × 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 10,000 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 IC₅₀ 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 300 nM, 10× above the SYK inhibitory IC₅₀, in the Millipore panel of 270 independent purified kinase assays. Kinases that were inhibited by >80% were then reevaluated to determine IC₅₀ using a 10-point concentration-response curve. Twenty-four kinases fit this criterion, and IC₅₀ values for each are shown in Fig. 1B, arranged in order of potency.

![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 IC₅₀. Kinases inhibited by greater than 80% were then tested against a 10-point PRT062070 concentration-response curve to determine IC₅₀. The IC₅₀ values for each of these kinases are shown in order of potency.](image-url)
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 downstream effectors, such as ERK1/2 (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 IC50 against ERK Y204 phosphorylation was 0.5 μM, with no appreciable inhibition of SYK Y352 phosphorylation at 4 μM (Fig. 2A). Consistent with the inhibition of SYK, PRT062070 reduced the ability of stimulated B cells to upregulate cell-surface expression of the early activation marker CD69 (IC50 = 0.11 μM). Inhibition of FcεRI-mediated basophil degranulation (IC50 = 0.12 μM) 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 upregulation in B cells at 4 μM (Fig. 2C). Furthermore, T-cell signaling after cross-linking of the T-cell antigen receptor or in response to PMA was unaffected by 4 μM PRT062070 (Fig. 2D), demonstrating specificity of action and lack of broad cross-reactivity to other pathways.

Inhibition of Cytokine Signaling and IL-4 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. IL-2 (JAK1/3) stimulation resulted in STAT5 Y694 phosphorylation in T cells. The PRT062070 IC50 values were 0.3 and 0.16 μM in CD4+ and CD8+ T cells, respectively (Fig. 3A). IL-4 (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 values of 0.58, 0.33, 1, and 0.92 μM, respectively (Fig. 3B). IL-6 (JAK1) stimulation leading to STAT3 Y705 phosphorylation in monocytes was inhibited with an IC50 of 0.35 μM (Fig. 3C), whereas...

Fig. 2. Inhibition of SYK-mediated signaling and functional responses in human whole blood. For each graph, the mean percent inhibition ± S.E.M. of the mean fluorescent intensity (MFI) normalized to vehicle control is reported on the y-axis and PRT062070 concentration (micromolar) is reported on the x-axis. (A) Human whole blood was stimulated with anti-IgD to measure LYN kinase activity (pSYK Y352; △, n = 7), SYK kinase activity (pERK Y204; ○, n = 8), and B-cell functional response (CD69 upregulation; ▲, n = 17). (B) FcεRI-mediated basophil degranulation in human whole blood (n = 4). (C) Human whole blood stimulated with PMA (n = 6) to induce protein kinase C (PKC)–dependent pERK Y204 (●) and CD69 upregulation (○) 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 (●) and pZap70 Y319 (○) in T cells. Horizontal bars in (C) and (D) depict the median.
granulocyte-macrophage colony-stimulating factor (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 use JAK1/JAK3 relative to JAK2 in cellular systems. IL-4 stimulation results in the upregulation of various cell surface activation markers in leukocytes. We therefore incubated human whole blood with IL-4 overnight in the presence of various concentrations of PRT062070 and measured the impact on functional responses in cells. In the presence of IL-4, B cells upregulated cell surface CD23, CD25, and CD69; the PRT062070 IC50 values for each were 1.5, 0.26, and 0.13 μM, respectively (Supplemental Fig. 1, left panel). Monocytes upregulated cell surface CD23 and CD25 expression, which were inhibited by PRT062070 with IC50 values of 0.23 and 0.36 μM, respectively (Supplemental Fig. 1, right panel).

BCR-induced cellular activation is potentiated by cytokine costimulation (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 IC50 = 0.11 μM; Fig. 2A) than would have been predicted from the potency of cellular SYK kinase inhibition after the same stimulation (ERK Y204 IC50 = 0.5 μM; 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 IC50 values 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 upregulation in human whole blood. SYK inhibition alone (PRT062070/BBIB057) resulted in full suppression of CD69 upregulation with an IC50 of approximately 0.3 μM. 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 Fig. 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 B-cell subtype (GC) diffuse large B-cell lymphoma (DLBCL) cell lines SU-DHL4 and SU-DHL6 were sensitive to SYK-selective (PRT060318; Fig. 4A, left) but not to pan-JAK (JAK inhibitor 1; Fig. 4A, right) 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) resulted in greater inhibition of cell viability relative to either
inhibitor alone. This conclusion was supported by the IC\textsubscript{50} values with nonoverlapping 95% confidence intervals (Fig. 4B). The 95% confidence interval for inhibition of Ramos cell viability was 0.49–0.72 μM with combined SYK plus JAK-selective inhibition versus 0.77–1.77 μM and 0.98–2 μM for SYK- and JAK-selective inhibition, respectively. Similarly, the 95% confidence interval for inhibition of Daudi cell viability was 0.85–1.15 μM with combined SYK plus JAK-selective inhibition versus 1.77–3.54 and 1.21–2.85 μM for SYK- and JAK-selective inhibition, respectively. PRT062070 (Fig. 4A, bottom right) 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 BCRs. 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 nontumor B cells. As shown in Fig. 5B, nearly 80% of the tumor B cells

**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), the pan-JAK inhibitor 1 (top right), combined SYK plus JAK-selective inhibitors (bottom left panel), and the dual SYK/JAK inhibitor PRT062070 (bottom right). Concentration in micromoles is displayed on the x-axis and percent inhibition of cell viability is shown on the y-axis. Data represent the mean ± S.E.M. of three independent experiments. (B) The table depicts the IC\textsubscript{50} and 95% confidence intervals for each treatment condition.
underwent apoptosis in the presence of PRT062070, whereas 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 after 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 7. Treatment with 0.5 mg/kg PRT062070 (attaining average $C_{\text{max}}$ plasma concentration at 2 hours of 0.18 mM) resulted in a nonstatistically significant trend toward reduced ankle inflammation, whereas significant reductions in inflammation were achieved with the 1.5, 3, and 5 mg/kg doses, with average $C_{\text{max}}$ plasma concentrations at 2 hours of 0.52, 0.58, and 1.49 mM, respectively. Inflammation was abolished at the 3 mg/kg dose and reversed relative to pretreatment levels at 5 mg/kg (Fig. 6A). Blinded histologic 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 histologic evaluations are shown in Supplemental Fig. 3.

As expected, PRT062070 treatment also affected anticolonagen antibody formation. Relative to the time of treatment initiation (ankle inflammation score of 1–2), circulating levels of anticolonagen 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 5 mg/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., 0.52–1.49 mM). To estimate the level of target inhibition achieved by these concentrations in rat blood, we performed IL-6 stimulations in the presence of a range of PRT062070 concentrations. The IC$_{50}$ for IL-6 signaling in CD3$^+$ T cells was 1.4 mM (Supplemental Fig. 4). Relating this to average $C_{\text{max}}$ plasma concentrations observed in the rat CIA model, 0.52 to 1.49 mM 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., 2013). PRT062070 inhibited mouse splenic B-cell activation in response to this antibody in vitro (Fig. 7A) and in vivo (Fig. 7B). Figure 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 15 mg/kg PRT062070 (third set of spleens). As shown in the bar graph of Fig. 7B, PRT062070 also suppressed upregulation of splenic B-cell surface CD80/86 and CD69 by 60%. The inhibition of splenomegaly was dose- and concentration-dependent (Fig. 7, C and D). Average $C_{\text{max}}$ plasma concentrations (2-hour post final dose) achieved in each dosing group is shown at the right of the graph in micromoles. (B) Corresponding mean modified Mankin histopathology scores (= S.E.M.; y-axis) determined in a blinded fashion are presented, with the treatment conditions shown on the x-axis. (C) and (D) Rat serum anticollagen antibody titers (micrograms per milliliter; y-axis) in which titers from naive immunized but noninflamed (score 0) and inflamed rats at scores of 1–2 and 4–8 are shown from a pilot study (C), and titers from naive and immunized rats treated with vehicle or various PRT062070 dose levels (milligrams per kilogram; x-axis) from a treatment study are shown (D). Horizontal bars depict the median. Statistically significant differences relative to vehicle control treated rats: *$P < 0.05$.

Discussion

We describe herein the preclinical characterization of the kinase inhibitor PRT062070. In the Millipore Kinase Profiler screen of 270 purified kinases, PRT062070 inhibited 24 kinases by more than 80% when tested at a concentration 10-fold above SYK $IC_{50}$. 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 physiologic ATP concentration. We observed very comparable potency in cellular assays measuring JAK/STAT, BCR/SYK, and FcɛRI/SYK, with limited to no activity against signaling pathways utilizing protein kinase C, lymphocyte-specific protein tyrosine kinase (LCK), and $\zeta$-chain–associated protein kinase 70 (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. These data are 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. However, we cannot rule out the possibility that inhibition of other kinases contribute to the overall 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; 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). IL-2, IL-4, and alpha interferon promote CLL survival in vitro, reportedly by inducing the upregulation 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 IL-4 induces upregulation 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 κ-light chain-enhancer of activated B cell–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 nontumor 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 IL-2, IL-6, and IL-15. Targeting SYK and JAK may therefore be relevant not only to tumor-intrinsinc prosurvival signaling but also to suppressing prosurvival 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 and 5; 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 IL-6 and/or IL-10, engaging an autocrine JAK/pSTAT3 signaling pathway that 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 antitumor activity in a DLBCL cell line relative to knockdown of each kinase alone (Ma et al., 2013). Consistently, broad antitumor 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.

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References


