

The selective Syk inhibitor P505-15 (PRT062607) inhibits B cell signaling and function in vitro and in vivo, and augments the activity of fludarabine in chronic lymphocytic leukemia

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**Running Title:** Syk inhibition augments fludarabine activity in CLL

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Chronic lymphocytic leukemia (CLL), Spleen tyrosine kinase (Syk), Non-Hodgkin's Lymphoma (NHL), Phosphorylation (phospo), B-Cell Receptor (BCR), Immunoglobulin (Ig)

## Abstract

B-cell receptor (BCR) associated kinases including spleen tyrosine kinase (SYK) contribute to the pathogenesis of B-cell malignancies. SYK is persistently phosphorylated in a subset of non-Hodgkin lymphoma (NHL) and chronic lymphocytic leukemia (CLL), and SYK inhibition results in abrogation of downstream kinase activity and apoptosis. P505-15 (also known as PRT062607) is a novel, highly selective and orally bioavailable small-molecule SYK inhibitor (SYK  $IC_{50}$  = 1nM) with anti-SYK activity that is at least 80-fold greater than its affinity for other kinases. We evaluated the pre-clinical characteristics of P505-15 in models of NHL and CLL. P505-15 successfully inhibited SYK mediated B-cell receptor signaling and decreased cell viability in NHL and CLL. Oral dosing in mice prevented BCR-mediated splenomegaly, and significantly inhibited NHL tumor growth in a xenograft model. In addition, combination treatment of primary CLL cells with P505-15 plus fludarabine produced synergistic enhancement of activity at nanomolar concentrations. Our findings support the ongoing development of P505-15 as a therapeutic agent for B-cell malignancies. A dose finding study in healthy volunteers has been completed.

## Introduction

Spleen tyrosine kinase (SYK) is a 72 kDa cytoplasmic tyrosine kinase primarily expressed in hematopoietic cells including B-cells. In B-cells, signal transduction is initiated by BCR activation via Src family kinase Lyn mediated phosphorylation of immune-receptor tyrosine-based activation motifs (ITAMs). This leads to the recruitment, autophosphorylation, and sustained activity of SYK and activation of a number of downstream effectors (Mocsai et al. 2010). Recent evidence suggests that B-cell malignancies including non-Hodgkin lymphoma (NHL) and chronic lymphocytic leukemia (CLL) can be driven by aberrant activity of cellular signaling pathways and by extrinsic factors from the micro-environment which interact with the BCR (Caligaris-Cappio and Chiorazzi, 2010). Increased SYK expression and/or activity has been implicated in a number of NHL histologies (Rinaldi et al., 2006; Tavolaro et al., 2006; Chen et al., 2008; Davis et al., 2010). In CLL, constitutive SYK activity, as well as activation after BCR cross-linking, have been described (Baudot et al., 2009; Gobessi et al., 2009). Increased expression of BCR associated kinases including SYK is associated with a shorter treatment free interval (Rodriguez et al., 2007), and SYK inhibition results in apoptosis (Baudot et al., 2009; Hoellenriegel et al., 2011) and disruption of chemokine activity (Rodriguez, et al., 2007; Hoellenriegel et al., 2011).

Targeting SYK has been explored using Fostamatinib disodium (R788), the pro-drug of R406. R788/406 is a SYK inhibitor ( $IC_{50} = 41nM$ ) found to have activity in a phase 2 study in NHL and CLL (Friedberg et al., 2010). However, R788/406 is known to have



significant off target effects including FMS related tyrosine kinase 3 (FLT-3), Lck, Janus kinase 1 and 3, and c-kit (Brasemann et al., 2006), which may be responsible for some of its activity (Davis et al., 2010). No further development of Fostamatinib or of another selective SYK inhibitor has been reported in lymphoid cancers. Therefore, the characterization of novel SYK inhibitors is warranted. Given the therapeutic potential of SYK inhibition and the need to develop SYK inhibitors without off target effects, we evaluated P505-15, a novel and highly selective ( $IC_{50} = 1$  nM) SYK inhibitor with anti-SYK activity that is 80-fold greater than its affinity for other kinases. P505-15 has been shown to potently inhibit SYK and BCR-dependent activation of normal B-cells (Coffey, et al., 2012) and has been shown to decrease CLL cell viability (Hoellenriegel et al., 2011). We aimed to further define the pre-clinical properties of P505-15 in NHL and CLL and its activity as a single agent or in combination with fludarabine in primary CLL samples, including those obtained from patients with poor risk biologic features.

## **Materials and Methods**

### **Chemical Structure and Kinase Profiling**

Synthesis and characterization of P505-15 [(4-(3-(2H-1,2,3-triazol-2-yl)phenylamino)-2-((1R,2S)-2-aminocyclohexylamino)pyrimidine-5-carboxamide acetate] (PRT062607) as well as its potency and selectivity for SYK have been reported (Coffey et al., 2012).

### **Cell lines and antibodies**

The human non-Hodgkin lymphoma B-cell lines SUDHL-4 (#ACC495), SUDHL-6 (#ACC572), and Karpas-422 (#ACC32) were obtained from DSMZ (Braunschweig, Germany); Toledo (#CRL-2631) and Ramos (#CRL-1596) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). All cells were maintained in RPMI media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (ATCC) and penicillin/streptomycin (Invitrogen), and maintained in a 37°C humidified tissue culture incubator. Antibodies included polyclonal goat F(ab)'<sub>2</sub> anti-human IgG and IgM (LifeTechnologies, Grand Island, NY); rabbit anti-human SYK, anti-human phospho-SYK (Y525/526), and alexafluor 488-conjugated anti-human phospho-ERK (Y204) (Cell Signaling Technologies, Inc., Danvers, MA); phycoerythrin-conjugated anti-human phospho-AKT (S473), fluorescein isothiocyanate-conjugated anti-human CD19, allophycocyanin-conjugated anti-human CD5, phycoerythrin-conjugated anti-human phospho-SYK (Y352), fluorescein isothiocyanate-conjugated anti-mouse CD80

and CD86, and allophycocyanin-conjugated anti-mouse CD45R/B220 (BD Biosciences, San Jose, CA).

### **SYK autophosphorylation**

Ramos cells ( $10^7$  cells per experiment) were pre-incubated for 30 min with or without P505-15 and then stimulated (30 min at 37°C) with 1µg/ml anti-IgM. Signaling was terminated by re-suspending cells in RIPA lysis buffer (50mM Tris 7.4, 1% NP40, 0.5% sodium deoxycholate, 150mM NaCl, 0.5mM EDTA), containing fresh protease (Roche) and phosphatase (Sigma) inhibitors, and incubated on ice for 1h. Following centrifugation, protein lysates were pre-cleared with protein A/G sepharose beads (Pierce, IL). Lysates were incubated overnight (4°C) with rabbit anti-SYK antibody, and precipitated with protein A/G sepharose beads. Washed beads were denatured, proteins resolved by 10% SDS-PAGE, immunoblotted with rabbit anti-SYK antibody, stripped and reblotted with rabbit anti-pSYK Y526/526 antibody.

### **Intracellular phospho-flow cytometry**

Ramos cells ( $0.5 \times 10^6$ ) were suspended in 200µl fresh media (RPMI plus 10% fetal calf serum) and pre-treated with vehicle or P505-15 (30min at 37°C). Cells were left unstimulated, or were stimulated with 1µg/ml goat F(ab)'2 anti-IgM (Life Technologies, Grand Island, NY) for 10 min. Ficoll purified ( $2 \times 10^6$ ) frozen viable CLL cells obtained from peripheral blood of CLL patients (n=7) were thawed at 37°C, washed in 10ml RPMI media plus 10% fetal calf serum by centrifugation, and re-suspended at  $10^6$  cells/ml in the same media. 200µl aliquots of cells were treated with vehicle or P505-15 for 30 min

prior to stimulation with 0.3mM H<sub>2</sub>O<sub>2</sub> (Reth 2002; Irish et al., 2006) (8.8M stock; Sigma, St. Louis, MO) followed by addition of 6µg of a 1:1 mixture of goat F(ab')<sub>2</sub> anti-human IgG and anti-human IgM for 10 min. Signaling was stopped by addition of 60µl of a 16% paraformaldehyde solution (Electron Microscopy Sciences, Hatfield PA) followed by 10 min incubation at room temperature. Fixed cells were washed twice in ice-cold phosphate buffered saline (PBS) by centrifugation, suspended in a 50% methanol solution in PBS pre-chilled to -20°C, and stored at 4°C overnight. Cells were stained for intracellular phosphoflow cytometry by washing the permeabilized cells twice in PBS containing 1% BSA, followed by incubation in the same buffer containing various antibodies, as indicated in the results, per manufacturer instructions. Cells were washed once more in PBS containing 1% BSA prior to FACS analysis, in which at least 2,000 events were collected using a BD Biosciences FACS Calibur. Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

### **Apoptosis**

Apoptosis was measured using the PE-conjugated monoclonal active caspase-3 antibody apoptosis kit (BD Biosciences). Cells were suspended in growth media (0.5 x10<sup>6</sup> cells/ml) and treated with the indicated concentrations of P505-15 or vehicle control for 72h prior to FACS analysis. In some experiments, SU-DHL6 (0.5 x 10<sup>6</sup> cells) were mixed with 100µl heparinized human whole blood. Samples were treated with 1µM P505-15 for 24h, and then surface stained with anti-human CD19 antibody prior to preparation for FACS analysis of active caspase-3.

### **In vitro and in vivo stimulation with anti-IgD**

Spleens were harvested from Balb/c mice and separated into a single cell suspension using a single cell strainer (BD Biosciences). Cells were collected in PBS containing 1% BSA by centrifugation, washed once in the same buffer, and resuspended in RPMI (containing 10% FCS) at  $10^6$  cells/ml. 190 $\mu$ l aliquots were then treated with various concentrations of P505-15 for 1h prior to stimulating overnight in a 37°C tissue culture incubator with 1 $\mu$ l anti-mouse IgD (EBiosciences, San Diego CA). Following stimulation, cells were stained with anti-CD80/86 FITC and anti-CD45R/B220 APC for 30 min at room temperature, washed once in PBS containing 1% BSA and resuspend in 300 $\mu$ l of the same buffer for collection of 2000 CD45 positive events by flow cytometry.

Balb/c mice (n=5 per group) received daily oral BID doses of vehicle (0.5% methylcellulose in water) or P505-15 (15mg/kg) for a total of 5 days. On study day 1, one hour after the first oral dose, mice received a single 200 $\mu$ l subcutaneous injection of control goat serum (Sigma) or anti-IgD serum (EBiosciences). On study day 5, mice were anesthetized with SC ketamine cocktail and exsanguinated via cardiac puncture. Spleens were weighed and then sectioned and stained with hematoxylin and eosin for histology.

### **Xenograft studies**

NOD/SCID mice were acclimated in-house at least three days prior to use. Ramos cells ( $3 \times 10^6$ ) were injected subcutaneously into the hind flank area of conscious mice using a 27 gauge needle in an injection volume of less than 0.5ml. Following injection, mice

were randomized into treatment groups (n = 15) and dosed twice daily by oral gavage with vehicle (0.5% methylcellulose in water) or 10, 15, or 20mg/kg P505-15. Body weights were obtained at least once per week and caliper measurements of tumors were determined twice per week beginning when palpable tumors were formed until the end of the study. Tumor volume was assessed by caliper measurement using a formula [maximum length x width x height x pi/6]. Twice daily dosing of vehicle or P505-15 continued until the vehicle or any treatment group exhibited tumors that exceeded 1.5 grams in size. At the time of termination (5 weeks post Ramos inoculation) the mice were anesthetized with a ketamine cocktail. A blood sample via cardiac puncture was obtained for CBC and plasma concentration determination and then the mice were euthanized via cervical dislocation. Tumors were excised, weighed, and then snap frozen in liquid nitrogen for determination of concentration of P505-15 in the tumor tissue. Statistical comparison of tumor weights across groups was performed using a t test.

### **Tumor and plasma concentration analysis**

Tumor-bearing mice were dosed twice daily for a total of twenty seven days. On the last day of dosing, tumor and plasma samples were collected at pre-dose, 1.5, 4, and 8 hr post-dose. Each tumor sample was homogenized in 3ml of saline per gram of tumor using the Kontes® Microtube Pellet Pestle® Rods and Motor (Kimble Chase, Vineland, NJ). Plasma and tumor samples were extracted and analyzed for P505-15 concentration using a liquid chromatography tandem mass spectrometer (LC/MS/MS). Pharmacokinetic

parameters of P505-15 in plasma and tumor were calculated using WinNonlin program, version 5.3 (Pharsight, Cary, NC).

### **Patient samples, viability assay, and synergy analysis**

Blood samples were obtained after written informed consent approved by the Institutional Review Board. CLL cells (n= 42) were purified using a ficoll gradient and added to wells ( $5 \times 10^4$  per well) containing 20% serum containing RPMI media plus  $\beta$ ME and four serial dilutions of P505-15 (10nM to 10 $\mu$ M) with or without fludarabine (10nM to 10 $\mu$ M). After 72h of culture, a tetrazolium-based cell viability assay (bioRad, MTS) was performed. The viability data were normalized to untreated controls and used to calculate half maximal inhibitory concentration ( $IC_{50}$ ) values. The Loewe surface/synergy was calculated using Combitool (Dressler et al., 1999). Chou median effects analysis and Bliss independence measures analysis were performed as described elsewhere (Chou 2010; Bliss 1939). Statistical significance was determined using the confidence interval.

## Results

### **P505-15 inhibits SYK activation and induces caspase dependent apoptosis in a subset of NHL cell lines**

To validate target inhibition, lysates from NHL cell lines stimulated with anti-IgM and treated with P505-15 (0, 2 $\mu$ M) were immunoblotted with antibodies specific for the SYK autophosphorylation sites (Y525/526). Significant reduction in SYK activity was observed (Figure 1A). To demonstrate inhibition of signaling more distal to the BCR/SYK complex, ERK (Y204) and AKT (S473) phosphorylation were monitored with or without SYK inhibition with P505-15 following BCR stimulation. Significant decreases in phosphorylation of both targets were seen (Figure 1B). Half maximal inhibition was observed at approximately 50nM, with complete inhibition of ERK and AKT phosphorylation at 250nM. As a specificity control, Lyn phosphorylation of SYK at Y352 was also measured. While inhibition was observed, the effect was not entirely concentration-dependent and complete inhibition could not be achieved (Figure 1B). Data from a representative experiment is shown in Figure 1C. These data are consistent with previous observations that P505-15 is a selective and potent inhibitor of Syk, with no appreciable activity against other BCR-related kinases (Coffey et al., 2012).

The effect of SYK inhibition on B-cell survival was measured by induction of caspase 3. The SU-DHL4, SU-DHL6, and Ramos B-cell lines express functional BCR, whereas the Toledo and Karpas-422 lines lack BCR and BLNK expression, respectively (Gabay, et al., 1999; Sprangers et al., 2006). P505-15 induced apoptosis only in the BCR-signaling



competent cell lines, consistent with observations made using other SYK inhibitors (Chen et al., 2008; Cheng et al., 2011), and highlighting the compound's specificity against its target kinase (Figure 2A). To compare the relative sensitivity of primary versus tumor B-cells, we performed mixing experiments in which SU-DHL6 was combined with human whole blood and treated with P505-15. Under these conditions, whereas the tumor B-cell line underwent apoptosis in response to SYK inhibition, primary B-cells did not (Figure 2B). Caspase 3 cleavage could be induced in primary B-cells upon treatment with the blasticidin (data not shown).

### **SYK inhibition protects against tumor formation in a mouse xenograft model**

The effect of SYK inhibition on Ramos tumor formation in a mouse xenograft model was assessed. Mice were dosed twice daily with 10, 15, or 20mg/kg P505-15 or vehicle control beginning on the day of tumor cell inoculation. Caliper measurements were initiated when tumors began to form, approximately three weeks post-tumor inoculation, and repeated every third day until termination of the study. The study was terminated when tumor weights began reaching approximately 1.5mg, at which time tumors were excised and weighed.

Tumor and plasma samples were evaluated for P505-15 levels on the last day of dosing. Immediately prior to the last dose on day 27, samples were collected to determine C<sub>min</sub>; following the last dose, samples were collected at 1.5h, 4h, and 8h, data from which were used for a composite PK analysis. More than dose-proportional increases in plasma and tumor C<sub>max</sub> and AUC (0-8h) and tumor C<sub>min</sub> were observed as the dose was increased, but a dose-proportional increase in plasma C<sub>min</sub> was

noted. Mean C<sub>max</sub> and AUC (0-8h) in plasma was at least 2-fold greater than that in tumor for all doses examined; however, mean nadir concentrations (C<sub>min</sub>) were higher in tumor than in plasma (Table 1A), suggesting accumulation of P505-15 in the tumor, and slower elimination from this compartment. The difference between plasma and tumor C<sub>min</sub> became more prominent as the dose was increased, as indicated by the increase in tumor/plasma ratios determined from C<sub>min</sub> (Table 1B). Tumor/plasma ratios determined from C<sub>max</sub> and AUC (0-8h) were similar across the various dose groups. Tumor concentrations of P505-15 at steady-state were sustained above 63, 176, and 325 nM over the entire dosing interval following 10, 15, and 20 mg/kg, respectively. The half life of P505-15 in mice following 1mg/kg IV dose was 3.9h .

Mice dosed with all three concentrations of P505-15 were protected from Ramos tumor growth *in vivo*. This was first evident from caliper measurements (Supplemental Figure 1), which revealed a reduced rate of tumor growth in the presence of P505-15. Upon study completion, mice were euthanized and tumors excised and weighed. Consistent with caliper measurements, a statistically significant reduction in average tumor weight was achieved in all dosing groups relative to vehicle control. Data are presented in Figure 3A. These data reveal that sub-micromolar concentrations of P505-15 can prevent tumor formation by an aggressive NHL cell line in mice. In a separate study (data not shown), treatment with P505-15 was initiated when tumor size was estimated by caliper measurement to be approximately 200mg. Mice were then randomized into treatment groups and treated with oral vehicle or P505-15 for the next 10 days, after which tumor

size approximated 1.5g and the study was terminated. Under these conditions P505-15 did not affect tumor progression. Cells derived from the extracted tumors did undergo apoptosis in response to in vitro treatment with P505-15, however, indicating that a non-responsive clone was not selected in vivo. Histological observation revealed that the tumors were minimally vascularized, and therefore may not be an appropriate model under these conditions. Irrespective, these data suggest that the efficacy observed under prophylactic treatment (Figure 3A) may be completely or in part due to inhibition of tumor engraftment.

Mice dosed with the P505-15 did not present with reduced numbers in any leukocyte subsets. In fact, the only effect observed was an increase in the number of lymphocytes in mice treated with 15mg/kg P505-15, which was not repeated in mice dosed with 10 or 20mg/kg (Figure 3B). The relative percent of each cell subtype analyzed was also unaffected. (data not shown).

### **SYK inhibition protects against BCR induced splenomegaly in mice**

Chronic BCR stimulation in secondary lymphoid organs appears to be an important driver of B cell survival and clonal expansion in certain B cell malignancies, particularly in CLL. To study this in vivo, we utilized an anti-mouse IgD that was previously shown to induce BCR signaling (Coffey et al., 2012) and immune cell activation in vivo following subcutaneous administration in mice (Mountz et al., 1987). We report here that P505-15 suppresses mouse B cell activation following stimulation with this anti-IgD (Figure 4A). Further, subcutaneous administration of this activating anti-mouse IgD results in rapid B cell expansion and splenomegaly in the animals. To determine the

effect of P505-15 on BCR-induced splenomegaly in vivo, groups of 5 mice were pre-treated with vehicle, or 15mg/kg compound for 1h prior to in vivo administration of anti-IgD antibody. Twice daily dosing was then continued for 4 days, with analysis of splenomegaly performed on day 5 post anti-IgD injection. In vivo stimulation with anti-IgD resulted in an approximate doubling of spleen weights, which was significantly reduced by treatment with P505-15 (Figure 4B). Spleens were sectioned and H&E stained to assess associated changes in morphology. Examples from mice treated with control goat serum (first panel), anti-IgD (second panel), and anti-IgD plus 15mg/kg P505-15 is presented in Figure 4C. Expansion of the marginal B-cell zone was observed in all anti-IgD stimulated mice treated with vehicle control, a phenomenon that was mostly corrected in mice upon treatment with the SYK inhibitor, as shown in the third panel. These data demonstrate that P505-15 can penetrate the mouse spleen following oral dosing in mice, and inhibit BCR signaling and clonal expansion in this environment.

### **P505-15 inhibits BCR mediated signaling and decreases cell viability in primary CLL and shows synergy when combined with fludarabine**

We next evaluated the ability of P505-15 to inhibit BCR-mediated SYK kinase activity in primary CLL samples (n=7). Representative flow cytometry data depicting BCR-mediated AKT phosphorylation in CD19/CD5 double positive CLL cells is shown in Figure 5A. Under these conditions, treatment with P505-15 resulted in a concentration dependent decrease in AKT phosphorylation, with complete inhibition of this target between 0.3 and 1 $\mu$ M in all samples tested (Figure 5B). Despite the potential for

increased BCR responsiveness in IgVh unmutated samples (Stevenson et al., 2011), decreased AKT phosphorylation after P505-15 treatment did not appear to be related to Zap 70 or IgVh mutational status (data not shown). The ability of P505-15 to inhibit AKT is of particular importance as agents that disrupt AKT activity have shown therapeutic promise in CLL (Lannutti et al., 2011).

Of the 42 primary CLL samples treated with P505-15 alone, activity was seen (defined as  $IC_{50} < 3 \mu M$  for each sample tested) in 15/42 (36%) samples. P505-15 single agent activity is consistent with prior work showing that P505-15 decreases CLL cell viability in the presence of anti-IgM mediated BCR activation or nurse-like cell co-culture (Hoellenriegel et al., 2011). We further characterized the effect of P505-15 across biologic subgroups. Importantly, significant activity was seen in samples obtained from patients with biologic features suggestive of high risk CLL which is characterized by the presence of having an unmutated variable region of the immunoglobulin heavy chain (IgVh), or deletion of chromosome 17p where p53 resides, or deletion of chromosome 11q (ATM gene). When further evaluating response ( $IC_{50} < 3 \mu M$ ) by poor risk groups, three of seven samples (43%) with 17p deletion, three of eight (38%) with 11q deletion, and four of seventeen (24%) with unmutated IgVh were found to be responsive to P505-15 treatment.

Fludarabine is a purine nucleoside analogue that is very active in CLL and continues to serve as the chemotherapy backbone in a number of chemotherapy regimens. However, it is associated with significant and at times long term myelotoxicity, opportunistic infections, and secondary malignancies. Furthermore, its efficacy in poor risk patients is

often lacking. Thus, combinations which incorporate drugs with novel mechanisms of action that could potentially decrease fludarabine exposure and toxicity while increasing activity are urgently needed. When primary CLL cells (n=20) were treated with P505-15 alone or in combination with fludarabine, cell killing was observed for both single agent and combinations (Figure 5C). When the highest concentration of fludarabine tested (10  $\mu$ M) was compared to lower concentrations (1  $\mu$ M) combined with P505-15 (100 nM and 1  $\mu$ M), equivalent cell killing was seen (Figure 5C; note dark shaded bars for comparison), indicating a fludarabine sparing effect when inhibiting SYK with P505-15. Further, synergy was detected by employing three independent analysis strategies; Loewe additivity, Bliss independence analysis, and Chou median effect analysis. By all three methods, 1  $\mu$ M fludarabine in combination with 1  $\mu$ M P505-15 resulted in synergistic cell kill. The Loewe and Chou analysis also detected synergism at 1  $\mu$ M fludarabine in combination with 0.1  $\mu$ M P505-15. The Loewe additivity analysis is represented in Figure 6.

## Discussion

Several receptors expressed by hematopoietic cells utilize SYK for signal transduction, including Fc receptors, BCR, integrins, and members of the lectin and selectin families (Turner et al., 2000; Mocsai et al., 2002; Rogers et al., 2005). In B-cells, signaling through the BCR is made possible by its association with Ig $\alpha$  and Ig $\beta$ , each bearing an ITAM domain that is phosphorylated by the Src family member Lyn (Jumaa et al., 2005). SYK engages the diphosphorylated ITAMs, a process that enhances its kinase activity, resulting in SYK autophosphorylation and tyrosine phosphorylation of multiple downstream substrates that ultimately regulate the cell's activation status, promoting survival and clonal expansion (Rolli et al., 2002). This signaling pathway is active in B-cells beginning at the transition from the pro- to pre-B-cell stage of development, when the newly formed pre-BCR is expressed. In fact, B-cell development arrests at the pro-B-cell stage in SYK knockout mice, and corresponds with a loss of mature B cells (Cheng, et al., 1995; Turner et al., 1995). Importantly, inducible loss of Syk for 10 days had no impact on mouse B cell populations (Wex et al., 2011), whereas at 4 weeks post inducible SYK depletion in adult mice a loss of mature B cells was observed, recapitulating the embryonic SYK knockout data (Ozaki et al., 2012). Inducible loss of the BCR (Lam et al., 1997) or Ig $\alpha$  (Kraus et al., 2004) also result in loss of peripheral mature B-cells in mice. These data suggest that normal B cells depend upon SYK for signals related to functional response and development, but not necessarily for survival.

However, the oncogenic potential of SYK has been described in a number of different settings. Clinically, SYK over-expression is reported in mantle cell lymphoma (Rinaldi,

et al., 2006) and the TEL-SYK fusion protein (Translocated ETS Leukemia) generated by a chromosomal translocation (t(9;12)(q22;p12)) leads to increased SYK activity and is associated with myelodysplastic syndrome (Kuno et al., 2001). SYK was also reported to mediate mTOR (mammalian target of rapamycin) survival signals in follicular, mantle cell, Burkitt's, and diffuse large B-cell (DLBCL) NHL (Leseux et al., 2006). Additional studies also suggest that SYK-dependent survival signals may play a role in DLBCL, mantle cell lymphoma and follicular lymphoma (Gururajan et al., 2006; Irish et al., 2006). In mice, a leukemia-like disease is induced by the adoptive transfer of bone marrow cells that express human TEL-SYK (Wossning et al., 2006), and BCR signaling is implicated in CLL tumor survival (Chiorazzi et al., 2005; Quiroga et al., 2009).

There is also evidence that primary lymphoma and human cell lines of NHL origin may depend on SYK signaling for survival. This was first reported by Monti et al (Monti, et al., 2005) who found that of 116 primary NHL tumor specimens analyzed for mRNA expression profiles, 43% clustered into a group characterized as over-expressing SYK and other components of the BCR signaling pathway. A subset of NHL cell lines was also reported to be dependent upon SYK expression in RNA interference studies (Davis et al., 2010). To determine if SYK was required for the survival of certain lymphomas, the protein tyrosine phosphatase PTP-RO, a negative regulator of SYK, was over-expressed in NHL cell lines. This resulted in the suppression of SYK signaling and induction of apoptosis (Chen et al., 2006).



Consistently, pharmacological inhibition of SYK activity induces apoptosis in a subset of NHL cell lines (Chen et al., 2008; Cheng et al., 2011). It was previously shown that SYK inhibition with R406 effectively inhibits SYK activity in NHL cell lines and demonstrates single agent activity in CLL including samples taken from patients with high risk features (Quiroga et al., 2009). Clinical activity of this compound in CLL/NHL trial was confirmed in a phase II trial (Friedberg et al., 2010); however, this agent has significant off target effects and phase III trials in oncology have not been pursued as yet in cancer. More recently, similar to other agents that target B-cell signaling, P505-15, a highly selective novel SYK inhibitor ( $IC_{50} = 1nM$ ) with greater than 80-fold selectivity for other kinases (Coffey et al., 2012), was demonstrated to inhibit BCR signaling. The inhibitor was also active in experimental systems which utilized chemokine and nurse-like cells to mimic the enhanced protection of CLL to cytotoxic chemotherapy or immunotherapy (Hoellenriegel et al., 2011).

Our studies confirm and extend previous observations by demonstrating that the capacity of SYK inhibitors to suppress BCR signaling and induce malignant B-cell apoptosis is not confined to cell lines or to cell cultures. Inhibition of BCR-mediated splenomegaly in mice provides strong evidence that P505-15 suppresses BCR signaling in vivo following oral dosing, and results from the Ramos xenograft model reveal that anti-tumor activity can be achieved at concentrations that do not affect primary leukocyte survival. These data were confirmed with the observation that tumor B-cells were uniquely sensitive to SYK inhibition in a mixture containing peripheral blood B-cells.

We also report here that P505-15 has modest single agent activity against CLL survival, but is synergistic with fludarabine at nanomolar concentrations. The observation that P505-15 concentrations in the range of 10 to 100nM enhance the effects of fludarabine (10nM to 1 $\mu$ M) demonstrates activity in excess of that expected from a purely additive effect (Tallarida, 2001). These findings are of particular importance given the well known dose limiting toxicity associated with repeated fludarabine treatment. Importantly, activity was seen in samples obtained from poor prognosis patients, including those harboring the 17p deletion, a group in tremendous need of additional therapeutic options. The activity seen in the 17p deleted samples is consistent with prior work suggesting that SYK mediated apoptosis is p53 independent (Baudot et al., 2009). The reliance on BCR mediated signaling in CLL with unmutated IgVh suggested that SYK inhibitors, especially in the presence of antigen, would preferentially exhibit increased activity in samples from patients with unmutated IgVh; however, this was not the case after P505-15 treatment. Furthermore, Zap 70 and CD38 expression, which are also markers of increased BCR responsiveness, did not correlate with P505-15 mediated growth inhibition.

Given its pre-clinical activity and specificity, especially when compared to previously studied SYK inhibitors, P505-15 is an attractive compound for clinical development. Additional studies aimed at defining the biological characteristics associated with SYK sensitivity are needed. Exploring additional P505-15 combinations including chemotherapy, monoclonal antibodies, or novel combinations of kinase inhibitors

targeting multiple branches of signaling pathways, may identify further therapeutic opportunities. A dose finding study using P505-15 in healthy volunteers has been completed and includes single and multiple dosing regimens.

### **Authorship Contributions**

Participated in research design: Spurgeon, Tyner, Druker, Coffey, Sinha

Conducted experiments: Fletcher, Burke, Coffey, DeGuzman, Baker, Hollenbach

Contributed new reagents or analytic tools: Pandey

Performed data analysis: Spurgeon, Loriaux, Betz, Coffey, Pak

Wrote or contributed to the writing of the manuscript: Spurgeon, Loriaux, Coffey, Sinha

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## Footnotes

- a) BJD is a Howard Hughes Investigator; J.W.T. is supported by grants from the William Lawrence and Blanche Hughes Fund, the Leukemia & Lymphoma Society, the V Foundation for Cancer Research, and the National Cancer Institute [4R00CA151457-03]. SES receives funding from the Medical Foundation of Oregon. RB is supported by funding from the Division of Hematology and Medical Oncology at Oregon Health & Science University. MML is supported by grants from the Leukemia and Lymphoma Society and National Cancer Institute [5R21CA159265]. P505-15 was provided by Portola Pharmaceuticals. GC, AB, FD, YP, DB, AP, SH, US are paid employees of Portola Pharmaceuticals and accordingly received support for portions of this research.
- b) Preliminary data was presented at the Annual Society of Hematology Meeting. *Blood* (ASH Annual Meeting Abstracts), Nov 2010;116: 2839.
- c) Reprint requests should be sent to Dr. Stephen E. Spurgeon (spurgeos@ohsu.edu), Mailcode UH73C, Multnomah Pavillion, Oregon Health & Science University. 3181 Sam Jackson Park Road, Portland, OR 97239.

## Figure Legends

**Figure 1: Suppression of BCR-induced SYK signaling.** **A)** Ramos cells were left unstimulated (No Stim) or stimulated with anti-BCR antibody (anti-IgM) in the presence or absence of 2 $\mu$ M P505-15. SYK autophosphorylation (pSYK Y525/526) is depicted in the top panel, and total SYK protein (SYK) in the bottom panel. **B)** Cells stimulated through the BCR and treated with the concentrations of P505-15 shown were evaluated by intracellular phospho-flow cytometry for the induction of ERK Y204 (black bars), AKT S473 (light grey bars), and SYK Y352 (dark grey bars). The mean fluorescent intensity (MFI) normalized to percent of vehicle control is plotted on the y-axis (mean + SD, n=3). After treatment of Ramos cells with P505-15, ERK (Y204) and AKT (S473) phosphorylation were inhibited. **C)** Representative experiment depicting the induction of ERK Y204 (top row y-axis), AKT S473 (bottom row y-axis), and SYK Y352 (x-axis) following treatment with the various concentrations of P505-15 shown.

**Figure 2: NHL cell lines are more reliant on SYK for survival than primary B-cells.** **A)** NHL cell lines treated with vehicle control, 1 $\mu$ M or 3 $\mu$ M P505-15. Histograms (representative of 2-3 experiments) depict induction of apoptosis via caspase 3 cleavage on the x-axis. The three sensitive cell lines are shown on the top row, and the two resistant lines on the bottom row. **B)** The side scatter (SSC) by CD19 scatter plots depict primary B-cell and SU-DHL6 gating strategies for human whole blood leukocytes (left plot) and human whole blood mixed with SU-DHL6 (right plot). Second row histograms depict primary B-cell or SU-DHL6 apoptosis following treatment with 1 $\mu$ M P505-15 for 24h. The dotted line represents vehicle control while the shaded grey histogram represents P505-15 treatment.

**Figure 3: SYK inhibition prevents NHL tumor formation in xenograft mice.** Mice were dosed twice daily with 10, 15, or 20mg/kg P505-15 or vehicle control beginning the day of tumor cell inoculation. **A)** Individual tumor weights (n = 13-15) were determined at 4 weeks post-inoculation for each treatment condition. Statistical differences relative to vehicle control are depicted as P values within the graph. **B)** Bar graphs depict normal blood cell counts (mean + SD, n=13-15) of mice treated with each concentration of P505-15 or vehicle control.

**Figure 4: Specific SYK inhibition prevents splenomegaly in a mouse model of chronic BCR stimulation.** **A)** Mouse splenocytes were isolated and treated with various concentrations of P505-15 as shown. Data represent inhibition of anti-IgD mediated B-cell activation (CD80/86 MFI) relative to vehicle control (mean + SD, n=4). **B)** Mice

were injected subcutaneously with goat serum (GS) or anti-IgD. Average spleen weight is graphed (mean + S.D. n=5) from mice treated as indicated. The asterisk indicates statistical difference ( $p < 0.05$ ) relative to anti-IgD mice treated with vehicle control (Vh). The subset photograph depicts two representative spleens from each group of mice treated with GS and vehicle control (first set), anti-IgD with vehicle (second set), and anti-IgD with 15mg/kg P505-15 (third set). C) Representative H&E staining of spleen sections from mice treated with GS (first panel), anti-IgD with vehicle (second panel), and anti-IgD with 15mg/kg P505-15.

**Figure 5: P505-15 inhibits BCR mediated signaling and when combined with fludarabine shows increased cell killing and is fludarabine sparing at nanomolar and low micromolar concentrations.** A) Representative BCR mediated AKT phosphorylation in CLL cells as shown by flow cytometry. The left and center panels depict gating on CD5+/CD19+ CLL cells. The histogram shows a representative (n=7) induction of AKT S473 following BCR ligation. B) P505-15 inhibits AKT phosphorylation following BCR ligation of CLL cells in a concentration-dependent manner (mean + SEM, n=7). C) CLL cells were treated with fludarabine alone, P505-15 alone, or in the combinations indicated. Percent inhibition of viability (mean and SEM, n=13) is plotted on the y-axis. Percent inhibition is P505-15 is dose dependent. The dark grey bars direct attention to cell killing in the presence of 10 $\mu$ M fludarabine alone (far left bar) relative to 1 $\mu$ M fludarabine combined with 1 $\mu$ M or 0.1 $\mu$ M P505-15 where equivalent cell kill was observed.

**Figure 6: P505-15 is synergistic with fludarabine at nanomolar concentrations in a CLL viability assay.** Cell viability data from P505-15 and fludarabine combination studies (n=18) were analyzed by CombiTool for determination of synergy. The combination effect is calculated by subtraction of the observed effect at certain concentrations of fludarabine and P505-15 from the calculated Loewe surface (Loewe surface in grey). The difference between observed activity and effect predicted from single drug controls (fludarabine or P505-15 alone by Loewe additivity model) are shown on 3-dimensional residual plots. Points that fall below the additive zero-interaction plane ( $z=0$ ) are synergistic (closed circles), since the observed inhibitory effect was greater than predicted. For example, points corresponding to fludarabine (1 $\mu$ M) + P505-15 (1 $\mu$ M) for all 20 pts and for fludarabine (1 $\mu$ M) + P505-15 (100nM) for 17/18 pts are below the Loewe surface demonstrating synergistic activity. For the point above the zero interaction plane using 0.01  $\mu$ M concentrations of P505-15 and fludarabine minimal growth inhibition was seen for either drug and thus no synergy could be appreciated.

**Table 1**

A)

<b>Determined from Plasma</b>				
<b>Dosing Regimen</b>	<b>Tmax (hr)</b>	<b>Cmin (nM)</b>	<b>Cmax (nM)</b>	<b>AUC (0-8hr; ng*hr/ml)</b>
10mg/kg BID	1.5	45	466	738
15mg/kg BID	1.5	69	893	1,671
20mg/kg BID	4.0	102	1,484	3,191

<b>Determined from Tumor</b>				
<b>Dosing Regimen</b>	<b>Tmax (hr)</b>	<b>Cmin (nM)</b>	<b>Cmax (nM)</b>	<b>AUC (0-8hr; ng*hr/ml)</b>
10mg/kg BID	8.0	63	143	353
15mg/kg BID	4.0	176	424	475
20mg/kg BID	4.0	325	656	1,453

B)

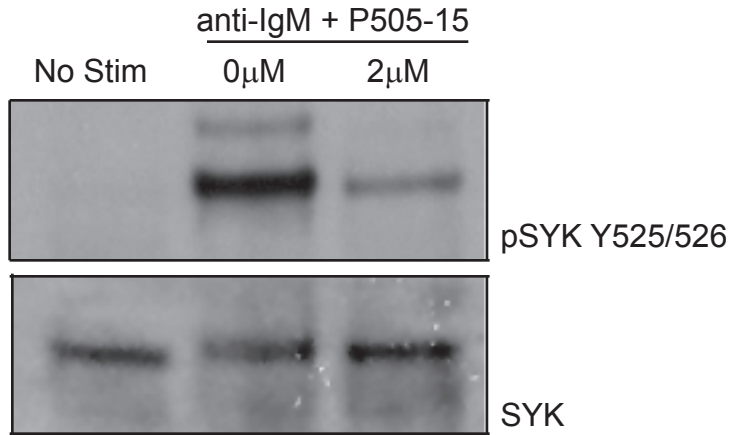
<b>Dosing Regimen</b>	<b>Tumor/Plasma Ratio</b>		
	<b>AUC based</b>	<b>Cmax based</b>	<b>Cmin based</b>
10mg/kg BID	<b>0.478</b>	<b>0.308</b>	<b>1.39</b>
15mg/kg BID	<b>0.284</b>	<b>0.475</b>	<b>2.55</b>
20mg/kg BID	<b>0.455</b>	<b>0.442</b>	<b>3.15</b>

**Table 1: Steady-state plasma and tumor pharmacokinetics following oral dosing of P505-15 in mice.** A) Plasma and tumor composite PK analysis from steady-state Cmin (collected 16h following the penultimate dose) and 1.5, 4, and 8h post-last dose on day 27. B) Tumor/plasma ratios are shown. Accumulation of P505-15 within the tumor is evidenced by ratios of greater than 1 at Cmin for each of the dose levels.

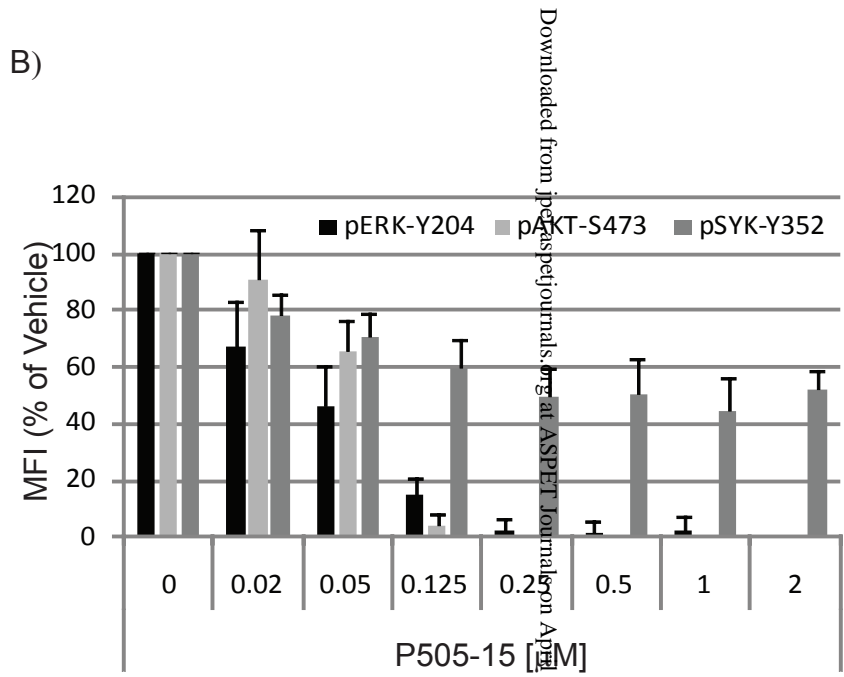


Figure 1

A)



B)



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C)

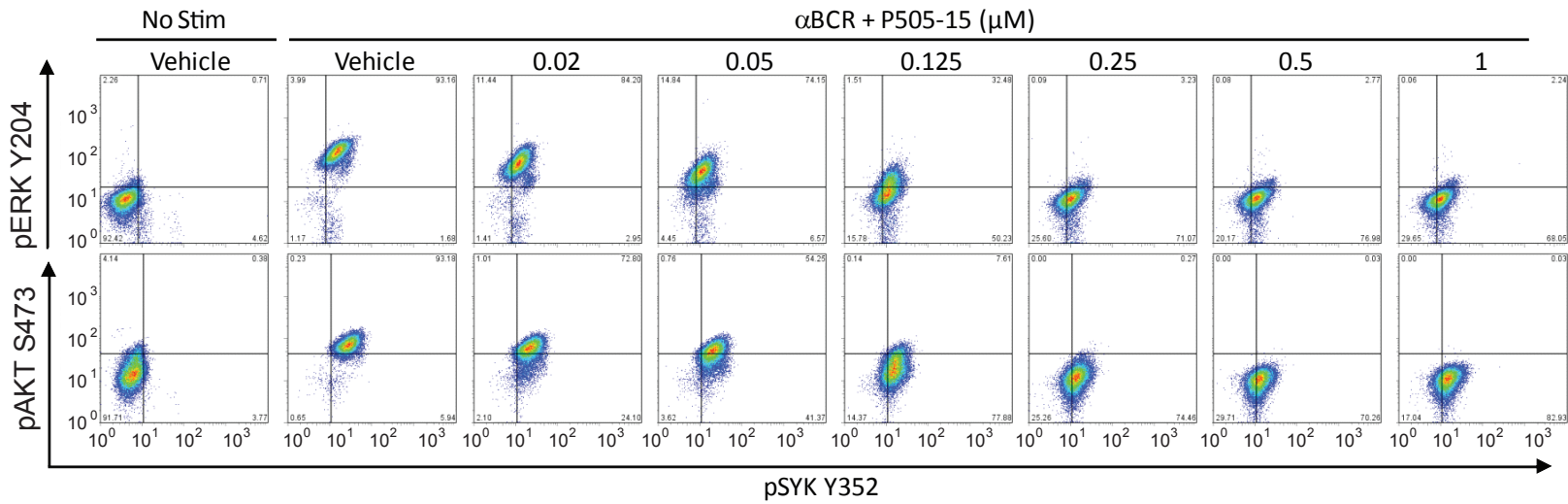
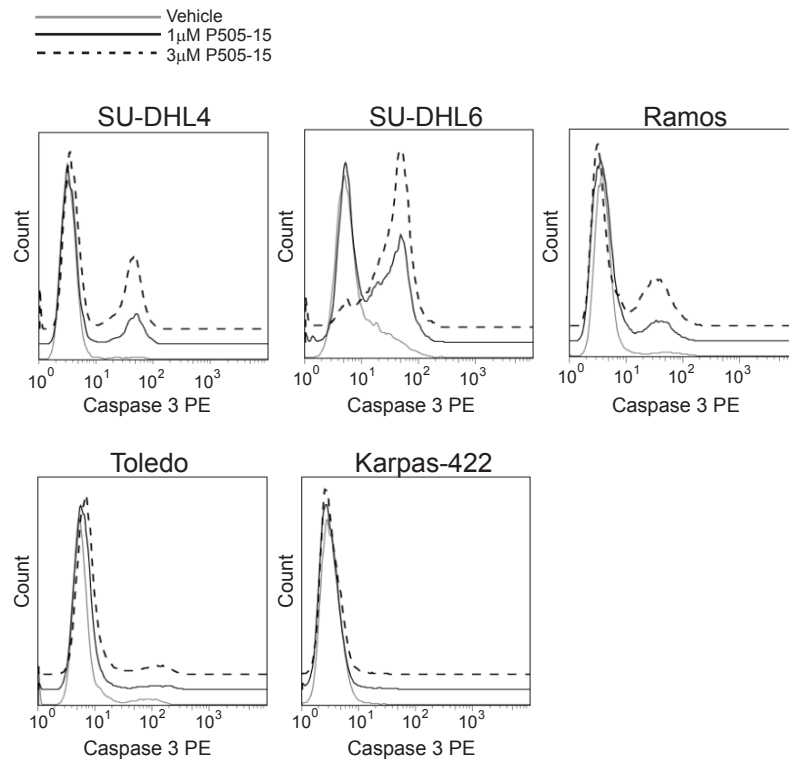


Figure 2

A)



B)

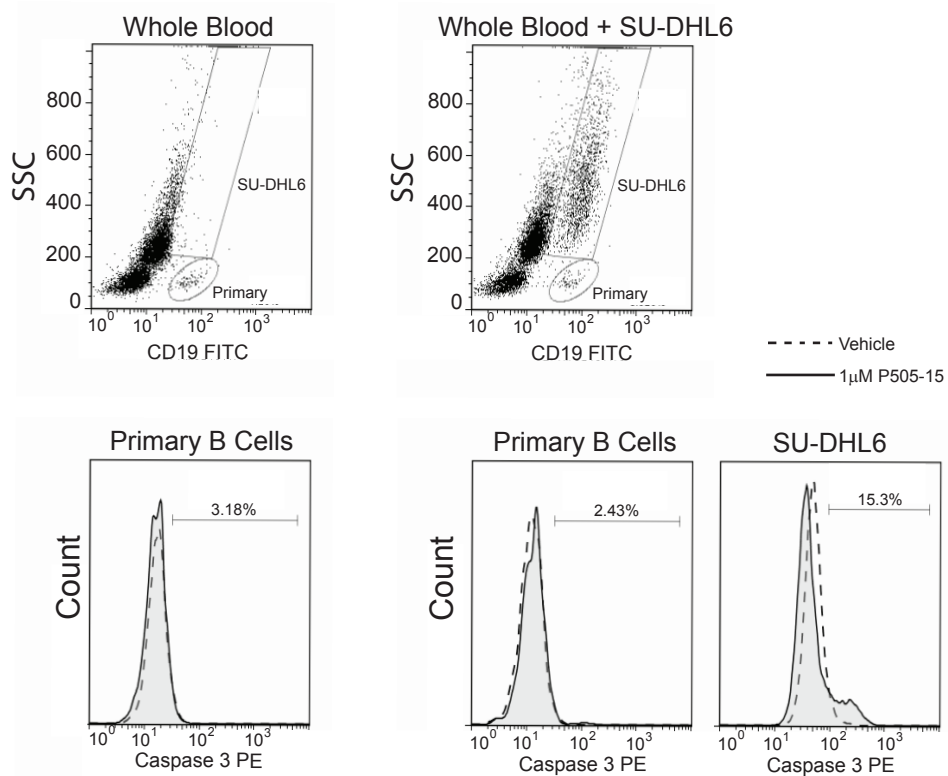
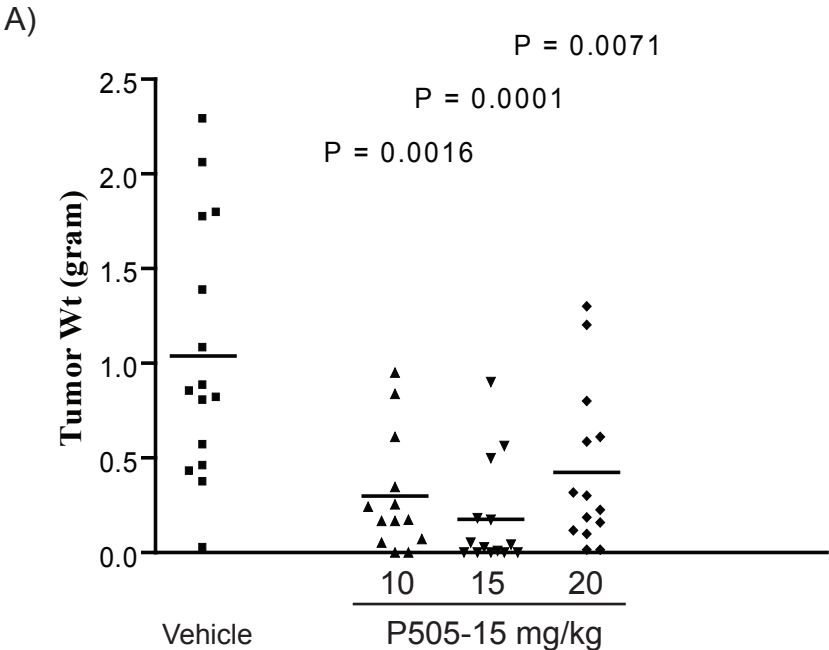


Figure 3



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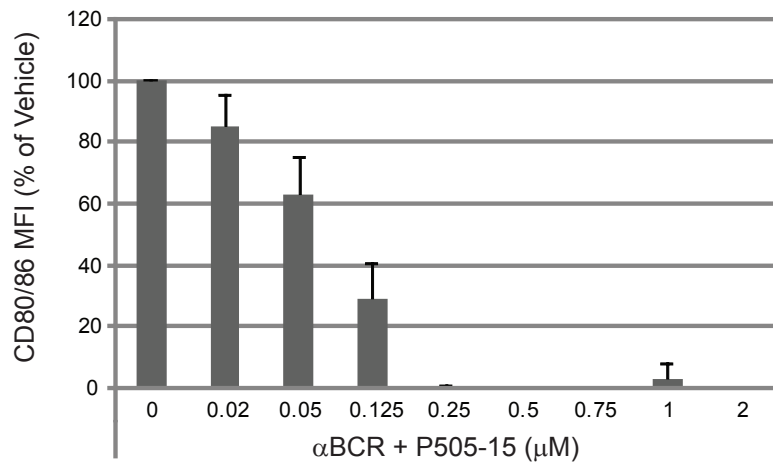
B)

Leukocytes (K/μl)	P505-15							
	Vehicle		10mg/kg		15mg/kg		20mg/kg	
	Average	Stdev	Average	Stdev	Average	Stdev	Average	Stdev
<b>White Blood Cells</b>	1.460	0.686	1.325	0.676	2.457	1.814	1.314	0.559
<b>Neutrophils</b>	1.204	0.606	1.016	0.556	1.429	0.736	0.926	0.397
<b>Lymphocytes</b>	0.143	0.048	0.232	0.254	0.740	0.903	0.210	0.215
<b>Monocytes</b>	0.059	0.035	0.044	0.023	0.114	0.093	0.076	0.051
<b>Eosinophils</b>	0.047	0.060	0.031	0.028	0.138	0.174	0.041	0.054
<b>Basophils</b>	0.007	0.018	0.004	0.007	0.036	0.063	0.006	0.009

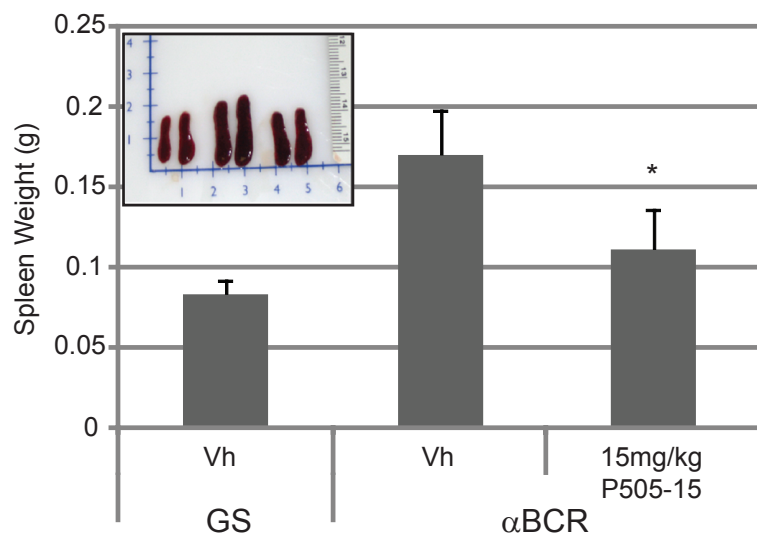


Figure 4

A)



B)



C)

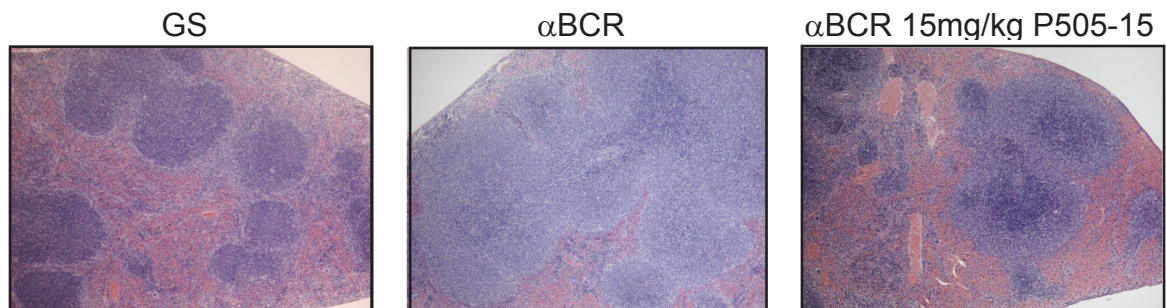
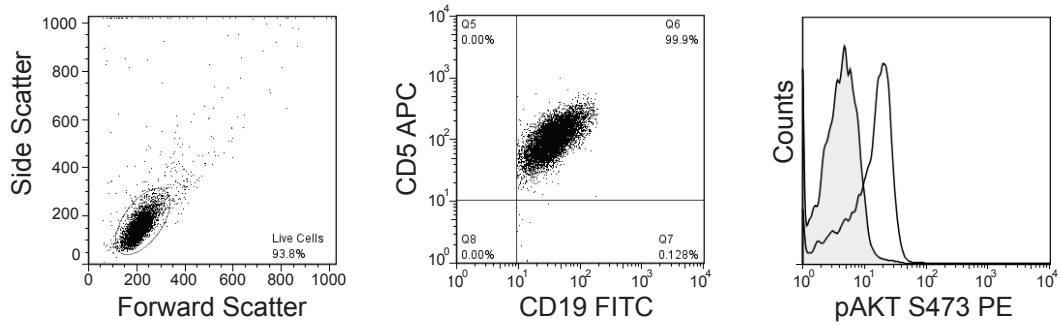
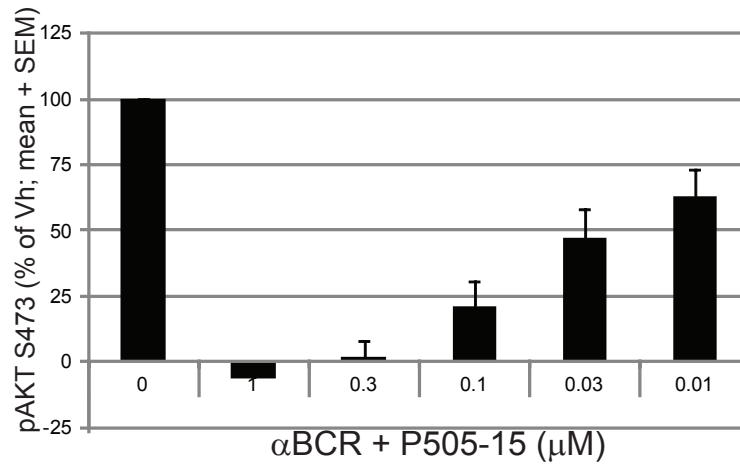


Figure 5

A)



B)



C)

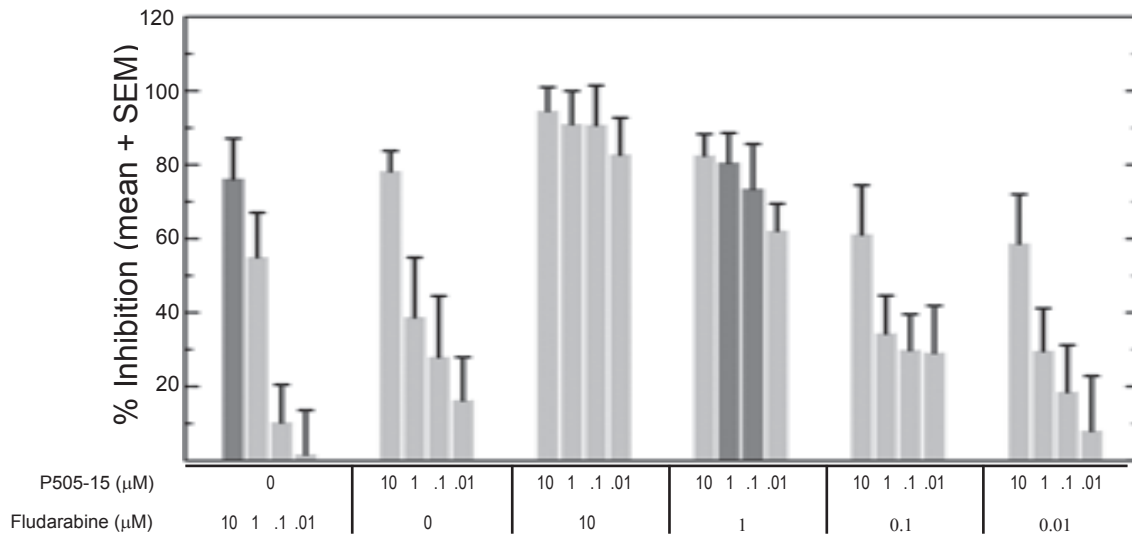


Figure 6

