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Selective Inhibition of BTK Prevents Murine Lupus and Antibody-Mediated Glomerulonephritis

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Autoantibody production and immune complex deposition within the kidney promote renal disease in patients with lupus nephritis. Thus, therapeutics that inhibit these pathways may be efficacious in the treatment of systemic lupus erythematosus. Bruton’s tyrosine kinase (BTK) is a critical signaling component of both BCR and FcR signaling. We sought to assess the efficacy of inhibiting BTK in the development of lupus-like disease, and in this article describe (R)-5-amino-1-(1-cyano Piperidin-3-yl)-3-(4-[2,4-difluorophenoxy]phenyl)-1H-pyrazole-4-carboxamide (PF-06250112), a novel highly selective and potent BTK inhibitor. We demonstrate in vitro that PF-06250112 inhibits both BCR-mediated signaling and proliferation, as well as FcR-mediated activation. To assess the therapeutic impact of BTK inhibition, we treated aged NZBxW F1 mice with PF-06250112 and demonstrate that PF-06250112 significantly limits the spontaneous accumulation of splenic germinal center B cells and plasma cells. Correspondingly, anti-dsDNA and autoantibody levels were reduced in a dose-dependent manner. Moreover, administration of PF-06250112 prevented the development of proteinuria and improved glomerular pathology scores in all treatment groups. Strikingly, this therapeutic effect could occur with only a modest reduction observed in anti-dsDNA titers, implying a critical role for BTK signaling in disease pathogenesis beyond inhibition of autoantibody production. We subsequently demonstrate that PF-06250112 prevents proteinuria in an FcR-dependent, Ab-mediated model of glomerulonephritis. Importantly, these results highlight that BTK inhibition potently limits the development of glomerulonephritis by impacting both cell- and effector molecule-mediated pathways. These data provide support for evaluating the efficacy of BTK inhibition in systemic lupus erythematosus patients. The Journal of Immunology, 2013, 191: 000–000.

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease that is characterized by autoantibodies recognizing nuclear components and for which there is currently significant unmet medical need. In SLE patients, autoreactive CD4+ T cells and B cells evade negative selection mechanisms and cooperate to produce affinity-matured autoantibodies that recognize dsDNA, RNA, and associated binding proteins (1). The autoantibodies produced by activated B cells bind target self-Ags and form immune complexes (ICs), which, in turn, deposit in tissues and promote organ damage via multiple mechanisms. The autoantibodies that recognize dsDNA, RNA, and associated binding proteins (1). The autoantibodies produced by activated B cells bind target self-Ags and form immune complexes (ICs), which, in turn, deposit in tissues and promote organ damage via multiple mechanisms. The autoantibodies that recognize dsDNA, RNA, and associated binding proteins (1). The autoantibodies produced by activated B cells bind target self-Ags and form immune complexes (ICs), which, in turn, deposit in tissues and promote organ damage via multiple mechanisms. The autoantibodies that recognize dsDNA, RNA, and associated binding proteins (1). The autoantibodies produced by activated B cells bind target self-Ags and form immune complexes (ICs), which, in turn, deposit in tissues and promote organ damage via multiple mechanisms. The autoantibodies that recognize dsDNA, RNA, and associated binding proteins (1). The autoantibodies produced by activated B cells bind target self-Ags and form immune complexes (ICs), which, in turn, deposit in tissues and promote organ damage via multiple
downstream activation of phospholipase Cy2 and Ca2+ release (13). These proximal signaling events promote activation of NF-κB- and NFAT-dependent pathways and expression of genes involved in proliferation and survival (14–16). Signaling via FcRy-associated receptors also promotes BTK-dependent proinflammatory cytokine production by cells such as macrophages (17). Because BTK signaling is critical for both B cell activation and FcR signaling, it represents an attractive therapeutic target for the treatment of SLE.

In this series of studies outlined later, we describe a novel BTK inhibitor (R)-5-amino-1-((1-cyanopiperidin-3-yl)-3-(4-[2,4-difluorophenoxy]phenyl)-1H-pyrazole-4-carboxamide (PF-06250112). We demonstrate that this compound exhibits high selectivity for BTK and potent inhibition of B cell activation mediated by BCR cross-linking and granulocyte degradation mediated by FcR cross-linking. We also describe the impact of therapeutic intervention with the BTK inhibitor PF-06250112 on SLE-like disease features in NZBxW_F1 mice and its effects on the systemic immune activation observed in this model. In addition, we examined the ability of PF-06250112 to prevent glomerulonephritis in an FcR-dependent, Ab-driven model. Our results demonstrate that inhibition of BTK signaling significantly impacts multiple key effector pathways that contribute to the pathogenesis of SLE, which has important implications for the treatment of SLE patients.

Materials and Methods

**Mice**

Six-week-old female NZBxW_F1 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were 26 wk of age at the beginning of the studies. Proteinuria scores were measured before initiating studies, and treatment groups were balanced according to the scores obtained. Female C57BL/6 mice were obtained from Taconic (Hudson, NY) and were 8–12 wk of age at the time of study initiation. All studies were approved by the Pfizer Institutional Animal Care and Use Committee.

**Drug formulation**

In vitro studies were performed using crystalline compound dissolved in DMSO. In vivo studies were performed using PF-06250112 prepared in a spray dried dispersion formulation delivered in hydroxypropylmethylcellulose acetate succinate.

**Western blots**

B cells were isolated from buffy coats by negative selection using RosetteSep Human B cell Enrichment Cocktail according to the manufacturer’s instructions (Stem Cell Technologies, Vancouver, BC, Canada). Isolated B cells were resuspended at 5 × 10^6/ml in PBS, and 5 × 10^6 cells were treated with different concentrations of PF-06250112 or DMSO as a vehicle control for 1 h at 37°C. Cells were then stimulated with 15 μg/ml anti-human IgM (Southern Biotech, Birmingham, AL) for 5 min at 37°C, lysed in cell lysis buffer containing protease and phosphatase inhibitors (Cell Signaling). Western blots were performed on cell lysates according to standard procedures using primary Abs recognizing BTK p-Tyr223 (Epitomics, Burlingame, CA), BTK p-Tyr551 (BD Pharmingen, San Jose, CA), spleen tyrosine kinase (Syk) p-Tyr525 (BD Pharmingen, San Jose, CA), spleen tyrosine kinase (Syk) p-Tyr562 (Cell Signaling Technology), total BTK (BD Transduction Laboratories, San Jose, CA), and total Syk (Cell Signaling Technology).

**Anti-IgE–mediated histamine release**

A total of 200 μl human whole blood was added to a 96-well plate. One microliter of compound or vehicle was added to each well, and the samples were then incubated at 37°C for 2 h. Cells were stimulated with 2 μg/ml anti-human IgE Ab for 30 min at 37°C. Triton X-100 was added to positive control samples to yield a maximal release measurement. Samples were spun down and plasma was assayed by ELISA to quantitate histamine levels.

**Human T and B cell proliferation assay**

B cells were isolated from buffy coats using RosetteSep Human B cell or CD4 T cell Enrichment Cocktail according to the manufacturer’s instructions (Stem Cell Technologies). A total of 2 × 10^5 B or CD4+ T cells were preincubated with BTK inhibitor or vehicle for 30 min at 37°C. B cells were stimulated with 30 μg/ml goat anti-IgM F(ab’)^2 (Southern Biotech). CD4+ T cells were stimulated with 1 × 10^5 anti-CD3/CD28 beads. Three days later, cells were pulsed with 1 μCi [3H]thymidine, harvested 18 h later, and counted.

**Whole-blood anti-IgE–mediated CD69 upregulation assay**

In brief, 500 μl whole blood and 1 μl compound or vehicle were added to a deep well plate and incubated at 37°C for 1 h. Twenty-five microliters of each sample was then stimulated with 20 μg/ml goat anti-IgG F(ab’)^2 and incubated at 37°C for 3 h (Southern Biotech, Birmingham, AL). Cells were then washed and stained with anti-CD19 APC and anti-CD69 PE Abs (BD Biosciences). RBCs were eliminated after staining by resuspending the cells in Fix/Lyse solution (BD Biosciences). Samples were analyzed on a flow cytometer as described later.

**In vivo treatments**

NZBxW_F1 mice were treated daily with PF-06250112 or vehicle by oral gavage. For anti-glomerular basement membrane-induced nephritis studies, mice were treated by oral gavage with PF-06250112 or vehicle 2 h before injection of mouse anti-rabbit IgG mAb and treatment was continued daily thereafter.

**Flow cytometry**

Single-cell suspensions of splenocytes were prepared, treated with RBC lysis buffer (Sigma), and then washed with FACS buffer (PBS plus 1% FBS). Cells were then incubated with purified anti-CD16/32 (Fc block; clone 2.4G2; BD Pharmingen), washed, and stained with primary Abs diluted in FACS buffer for 30 min at 4°C. Abs used in these studies included anti-T and B cell–activating Ag “GL7” (clone GL7), CD4 (clone GK1.5), CD45 (clone 30-F11), AA4.1 (clone AA4.1), CD21 (clone 7E9), CD23 (clone B3B4), CD3 (clone 145-2C11), CD8a (clone 53-6.7), CD138 (clone 281-2), B220 (clone RA3-6B2), CD19 (clone 1D3), IgM (II/41), IgD (11-26c.2a), CD44 (clone IM7), and CD62L (clone MEL-14). Cells were also stained with live/dead Fixable Aqua dead cell stain (Invitrogen), as per the manufacturer’s directions. At least 100,000 events were counted using either an LSRII or Fortessa flow cytometer (Becton Dickinson) and analyzed using FlowJo software (Tree Star). All spleen samples were first gated on ‘Live’ (Aqua Live Dead-) singlets. CD4+ T cells were additionally gated as follows: CD3+, B220−, CD4+, CD8− and assessed for levels of CD44 and CD62L. Germlinal center B cells were identified as CD5−, B220+, Fas+, IgM+, IgD−. Absolute numbers of B cells in blood were calculated by determining the percentage of lymphocytes bearing the cell-surface phenotype of interest by flow cytometric analysis and multiplying by the absolute number of lymphocytes in blood samples obtained by a Cell-Dyn 3700 automated hemolyzer (Abbott, Abbott Park, IL).

**ELISPOT**

For detection of anti-dsDNA Ab-secreting cells (ASCs), ELISPOT plates (Millipore) were coated with methyl-BSA (2 μg/ml; Sigma), washed with PBS containing 0.05% Tween 20, and incubated overnight with double-stranded calf thymus DNA (2 μg/ml; Sigma). Plates were blocked with RPMI 1640 containing 10% FCS (Sigma) for 1 h at 37°C. Splenocytes were harvested and single-cell suspensions were prepared in RPMI 1640 with 10% FCS. Cells were plated and incubated overnight at 37°C. Anti-dsDNA–specific ASCs were detected using alkaline phosphatase–conjugated goat Abs specific for mouse IgG (Southern Biotechnological) and developed with NBT/5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (KPL, Gaithersburg, MD). Spots were counted using an AID ELISPOT reader and Elispot 5.0iSPot software.

ELISPOT plates were coated with unlabeled goat anti-mlg (H+L; Southern Biotech) in PBS at 1 μg/ml and incubated overnight, to detect total IgM or IgG ASCs. Splenocytes were plated in RPMI 1640 containing 10% FCS and incubated for 4 h at 37°C. Detection was performed with alkaline phosphatase–conjugated goat Abs specific for mouse IgM or IgG. Plates were developed and analyzed as described earlier.

**Anti-dsDNA ELISA**

Serum anti-dsDNA IgG Ab levels were measured by ELISA as described previously (18). In brief, Immulon 1B plates (Thermolab Systems, Waltham, MA) were ultraviolet irradiated overnight and then coated with 2 mg/ml calf thymus DNA (Sigma, St. Louis, MO) for 1 h at room temperature. Plates were blocked with PBS plus 1% BSA, diluted serum samples were added,
and bound Ab was detected with an HRP-conjugated goat anti-mouse IgG polyclonal Ab (Southern Biotech, Birmingham, AL). Plates were developed using 3,3',5,5'-tetramethylbenzidine (KPL, Gaithersburg, MD), and reactions were stopped with 2 N sulfuric acid. Absorbances were read at 450 nm using a SpectraMax Plus 384 microplate reader ( Molecular Devices, Sunnyvale, CA) and analyzed using SoftMax Pro software. Fold change in anti-dsDNA Ab levels is reported and was calculated by dividing matched poststudy titers by pretreatment titers.

**Serum immunoglobulin titers**

Serum Ig titers were measured using a multiplexed isotyping assay (Meso Scale Discover, Gaithersburg, MD) according to the manufacturer’s recommendations. Serum samples were diluted 1:50,000 in PBS containing 1% BSA.

**Histology and immunohistochemistry**

At necropsy, kidneys were sampled using a systematic unbiased approach wherein each kidney was divided into four coronal sections; two sections were immersed in 10% neutral buffered formalin for histopathology; one section was placed into optimum cutting temperature medium and quickly frozen for assessing IgG and C3 immunohistochemistry expression; and one section was snap-frozen in liquid nitrogen for RNA profiling. Appropriation of coronal tissue sections for end-point analysis was randomized across all subjects.

Paraffin-embedded kidney was sectioned serially at 4 μm, and sections were stained with H&E or with periodic acid–Schiff (PAS) reagent with a hematoxylin counterstain. Staining for IgG and C3 expression was performed on kidney sections cut at 4 μm. Sections were cut in cold acetone 4°C, air-dried overnight, and rehydrated in TBS. Endogenous peroxidase was quenched with 3% H2O2 in 70% acetone-free methanol, rinsed with TBS, and stained in a DakoCytomation Autostainer (Carpinteria, CA; with either goat anti-mouse IgG or goat anti-mouse C3 polyclonal Abs (ICN/ Cappel, Irvine, CA). Hematoxylin was used as the counterstain. Images were captured with a Nikon Eclipse 50i microscope (Nikon, Melville, NY) and NIS Elements BR 2.20 software.

**Histopathologic scoring**

A board-certified veterinary pathologist blindly examined and semiquantitatively scored kidney sections as follows: H&E-stained sections for inflammatory foci and glomerular pathology, PAS-stained sections for glomerular pathology and proteinaceous (hyaline) casts, and IHC-stained sections for IgG and C3 expression. The following scoring schemes were used: inflammatory foci: 5 = too numerous to count; 4 = widespread distribution of all; 3 = numerous foci scattered throughout tissue, including interstitium, intraglomerular, perivascular, periglomerular, and peripelvic, with lymphoid follicle formation; 2 = limited distribution of inflammatory foci, with or without lymphoid follicle formation; 2 = leukocytic infiltrates are primarily perivascular and composed of dense foci; 1 = <25 leukocytic clusters of up to 25 lymphocytes; 0 = minimal leukocytic infiltration; cast score: 5 = 2000 mg/dl. Trained staff obtained proteinuria scores in a blinded manner. Proteinuria was quantitatively scored kidney sections as follows: H&E-stained sections for inflammatory foci and glomerular pathology, PAS-stained sections for glomerular pathology and proteinaceous (hyaline) casts, and IHC-stained sections for IgG and C3 expression. The following scoring schemes were used: inflammatory foci: 5 = too numerous to count; 4 = widespread distribution of all; 3 = numerous foci scattered throughout tissue, including interstitium, intraglomerular, perivascular, periglomerular, and peripelvic, with lymphoid follicle formation; 2 = limited distribution of inflammatory foci, with or without lymphoid follicle formation; 2 = leukocytic infiltrates are primarily perivascular and composed of dense foci; 1 = <25 leukocytic clusters of up to 25 lymphocytes; 0 = minimal leukocytic infiltration; cast score: 5 = 2000 mg/dl. Trained staff obtained proteinuria scores in a blinded manner.

**Proteinuria**

Proteinuria was measured using Albustix (Bayer) and scored on a scale of 0 to 5; 0 = none; 1 = trace; 2 = 30 mg/dl; 3 = 100 mg/dl; 4 = 300 mg/dl; and 5 = 2000 mg/dl. Train staff obtained proteinuria scores in a blinded manner. Four of 10 animals in the vehicle-treated group experienced development of severe proteinuria while on study and required euthanization before study completion. One of 10 animals in the vehicle-treated, 10 mg/kg PF-06250112–treated, and 30 mg/kg PF-06250112–treated groups lost ≥20% of their body weight and required euthanization as per Institutional Animal Care and Use Committee regulations. Kidneys from all animals that required euthanization were snap-frozen and assessed histologically upon study completion. Animals euthanized as a consequence of weight loss appeared otherwise healthy at the time of euthanization and did not exhibit evidence of renal pathology based on histopathologic analysis or urinary dipstick measures. Proteinuria scores for mice euthanized on-study were included in subsequent weeks measurements and were derived from scores obtained just before euthanization.

**Anti–glomerular basement membrane Ab-induced nephritis**

Ab-induced nephritis was established using previously described methods (8). In brief, rabbit anti–glomerular basement membrane (anti-GBM) polyclonal Ab was generated and the IgG fraction was purified over a Protein A column. Reactivity of this purified material to the noncollagenous domain of type IV collagen (Chondrex, Redmond, WA) was confirmed by ELISA (data not shown). Mouse anti-rabbit IgG (mIgG2a) was produced from hybridoma CRL-1753 (American Type Culture Collection, Manassas, VA) and purified over a Protein G column. Mice were first i.v. injected with 1.0 mg anti-GBM Ab and the following day were i.p. injected with 0.5 mg purified mouse anti-rabbit IgG mAb to induce Ab-mediated nephritis.

**Immunofluorescence**

Kidneys were harvested, decapsulated, and placed in a 30% sucrose/PBS solution before freezing in optimum cutting temperature. Five-micrometer tissue sections were cut on a cryostat, fixed in acetone, and stained with goat anti-mouse C3/FITC (MP Biomedicals, Solon, OH), goat anti-mouse IgG: Alexa 488 (Invitrogen) polyclonal Abs diluted in wash buffer (DAKO, Carpinteria, CA) containing 10% FBS. Slides were washed in wash buffer, covered in mounting medium containing DAPI (Invitrogen), and coverslipped. Images were captured on a Nikon Eclipse E800 microscope (Melville, NY) equipped with a Spot RT Slider Camera and Spot Software V4.7 (Sterling Heights, MI).

**Statistical analysis**

Statistical analysis was performed by one-way ANOVA or nonparametric Kruskal–Wallis analysis where indicated. The p values were determined by post hoc testing of all treatment groups. Analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA).

**Results**

PF-06250112 potently and selectively inhibits BTK

Through lead optimization efforts, PF-06250112 was identified as a potent, orally bioavailable, small-molecule inhibitor of BTK (Fig. 1A). PF-06250112 forms a covalent but reversible adduct with BTK through modification of a cysteine (Cys) residue (Cys481) proximal to the ATP binding pocket as demonstrated through crystal structure analysis and mass spectrometric characterization (data not shown). In in vitro enzyme assays, PF-06250112 inhibited BTK in a time-dependent manner (IC50 = 0.5 nM, Kcat/Km = 120,000 lM/s) and it demonstrated slow-off kinetics (T1/2 = 7 h; Fig. 1B and data not shown). General kinome selectivity was high, including closely related Src family kinases (Lyn, Src, and lymphocyte-specific protein kinase [Lck]; 2200- to 4000-fold selective; Supplemental Table I). Besides BTK, 10 additional kinases retain a highly conserved active site Cys at the homologous position as found in BTK. Within this kinase family, PF-06250112 showed comparable potency toward BMY nonreceptor tyrosine kinase (IC50 = 0.9 nM) and TEC (IC50 = 1.2 nM) as BTK; however, high selectivity was achieved for other members including IL-2-inducible T cell kinase and JAK3 (>10,000-fold selective; Supplemental Table I).

PF-06250112 inhibits BCR-mediated B cell signaling, activation, and proliferation

BCR cross-linking initiates an intracellular signaling cascade that promotes BTK-dependent proliferation of B cells (19). We assessed the functional impact of inhibiting BTK activity on signaling via the BCR. As expected, treatment with PF-06250112 did not impact phosphorylation of Syk, which occurs proximal to BTK activation (Fig. 1C). Phosphorylation of BTK subsequently occurs via a two-step process with phosphorylation of Tyr551 by kinases such as Syk and resultant autophosphorylation of BTK at Tyr223 (13, 20). Phospho-BTK subsequently leads to activation of phospholipase Ca2 and downstream calcium flux via the production of second
Results are representative of seven independent experiments. (Fig. 1C) By contrast, treatment with PF-06250112 inhibited p-Tyr223 in a dose-dependent manner (Fig. 1C). Moreover, treatment with PF-06250112 profoundly suppressed the production of inositol monophosphate in Ramos cells, a human B cell line, with an IC50 of 12 nM (Fig. 1D).

We next assessed the effect of PF-06250112 on cellular responses. Purified primary human B cells stimulated through the BCR undergo robust proliferation in vitro, and treatment with PF-06250112 significantly inhibited this response with an IC50 of 2.5 nM (Fig. 1E). Conversely, PF-06250112 only modestly inhibited proliferation of primary human T cells stimulated with anti-CD3 and anti-CD28 Abs, and high micromolar concentrations of the compound were required to observe this effect (Fig. 1E). Similar results were obtained with purified murine B cells (IC50 = 0.6 nM) and T cells (IC50 > 10 μM; data not shown). To quantify inhibition of B cell activation in a more physiologic setting, we determined the potency of PF-06250112 in a human whole-blood assay by monitoring CD69 upregulation on B cells in response to treatment with anti-IgD Ab (23). Pretreatment with PF-06250112 prevented anti-IgD–mediated CD69 upregulation on B cells with an IC50 of 23 nM (Fig. 1F; Supplemental Fig. 1A). We also assessed the ability of PF-06250112 to inhibit anti-IgE–mediated histamine release in human whole blood. Anti-IgE–mediated degranulation of mast cells and basophils is dependent on FcεRε and signaling via BTK (24, 25). PF-06250112 inhibited anti-IgE–mediated histamine release in human whole blood with an IC50 of 68 nM (Fig. 1G). Collectively, these data indicate that PF-06250112 potently inhibits BTK signaling and its associated cellular response pathways.

**PF-06250112 prevents the development of renal pathology in NZBxW_F1 mice**

NZBxW_F1 mice spontaneously develop lupus-like disease features that include systemic immune activation, anti-dsDNA autoantibody production, and glomerulonephritis (26). We sought to assess the ability of PF-06250112 to impact lupus-like disease features. We first characterized the pharmacokinetic properties of the PF-06250112 in NZBxW_F1 mice. Maximal circulating levels of PF-06250112 were observed at 4 h postdosing and the compound exhibited a T1/2 of ∼7 h (Supplemental Fig. 2A, 2B). We treated NZBxW_F1 mice with vehicle or 3, 10, or 30 mg/kg PF-06250112 starting at 26 wk of age, at which time all animals have elevated anti-dsDNA titers and severe proteinuria begins to develop (data not shown). Treatment with PF-06250112 was continued for 12 wk. Drug levels were examined every other week in each treatment cohort with sampling occurring at ∼4 h postdosing. The drug exposures exhibited dose proportionality and were maintained at a constant level throughout the study course (Supplemental Fig. 2C).

Upon completion of the study, kidneys were collected and assessed for the extent of renal pathology that had developed. Histopathologic analysis of kidneys obtained from vehicle-treated animals revealed extensive mononuclear leukocytic infiltrates located primarily around the renal pelvis, which is the funnel-shaped expansion of the upper end of the ureter into which the renal calices

Results are representative of three unique donors. (G) Graph shows the inhibition of histamine release after cross-linking treatment of human whole blood with anti-IgE Ab. Symbols show the mean value obtained from seven donors, and error bars indicate the SEM.
open (Fig. 2A). In severely diseased animals, lymphoid follicle formation was apparent in the peripelvic infiltrates, and leukocytic infiltrates often surrounded arteries and glomeruli, and infiltrated the interstitium (Fig. 2A and data not shown). Treatment with PF-06250112 significantly reduced inflammatory infiltrates in a dose-dependent manner with a near absence of infiltrates observed at the 30 mg/kg dose (Fig. 2A, 2B). Glomeruli in vehicle-treated animals also exhibited morphologic changes, appearing enlarged and hypercellular (Fig. 2C, 2D). Mesangial matrix accumulation was apparent, and glomeruli often exhibited capsular fibrosis and formation of epithelial crescents. Deposits of IgG and the complement component 3 (C3) were also readily observed in glomeruli from vehicle-treated animals (Fig. 2E–H). Strikingly, glomerular injury was significantly reduced by PF-06250112 treatment at each of the doses tested (Fig. 2C, 2D). Both IgG and C3 deposition were also significantly reduced in mice treated with 10 or 30 mg/kg PF-06250112 (Fig. 2E–H).

To assess the extent of kidney damage using a functional end point, we measured urinary protein levels every other week throughout the study course. Severe proteinuria began to develop in vehicle-treated animals starting at 26 wk of age and was defined as protein concentrations that were $\geq 300 \text{ mg/dl}$ (Fig. 3A). By the end of the treatment period at 38 wk of age, 70% (7/10) of vehicle-treated animals developed severe proteinuria (Fig. 3A). By contrast, none of the animals treated with PF-06250112 developed severe proteinuria during this time frame (Fig. 3A). We measured terminal microalbumin levels by urinalysis as an independent measure of kidney damage. Microalbumin levels were significantly lower in samples obtained from mice treated with PF-06250112, with similar results observed by dipstick analysis and quantification of renal proteinaceous casts (Fig. 3A–C). These results indicate that treatment with PF-06250112 can profoundly inhibit the development of glomerulonephritis and the resultant proteinuria.

**PF-06250112 inhibits the accumulation of activated B and T lymphocytes in NZBxW_F1 mice**

We next examined the impact of treatment with PF-06250112 on the spontaneous accumulation of activated B cell populations in NZBxW_F1 mice. Spleens obtained from vehicle-treated 38-wk-old animals contained large numbers of germinal center B cells and plasma cells (Fig. 4A, 4B). Treatment with PF-06250112 profoundly suppressed spontaneous germinal center formation with a 25-fold reduction in germinal center B cell numbers ob-

![FIGURE 2.](http://www.jimmunol.org/)
A center phenotype (gated; Fas+ GL7+) is are gated on “Live” singlet CD3 in the various treatment groups. Plots (shown for the indicated treatments.

Sections. Significance bars indicate quantitatively based on histopathologic analysis of H&E-stained kidney sections. Significance bars indicate $p < 0.05$, ANOVA with Bonferroni posttest. Error bars indicate the SEM throughout the figure. (C) Proteinaceous casts were scored semi-quantitatively based on histopathologic analysis of H&E-stained kidney sections. Significance bars indicate $p < 0.05$ (one-way ANOVA with Bonferroni posttest).

BTK INHIBITION PREVENTS GLOMERULONEPHRITIS

FIGURE 3. PF-06250112 prevents the development of proteinuria in NZBxW_F1 mice. (A) Graph shows the percentage of animals ($n = 10/ group) with severe proteinuria, which was defined as $>300$ mg/dl protein. (B) Microalbumin levels were quantitated at the end of the study, and the results are shown for each of the treatment groups. Significance bars indicate $p < 0.05$, ANOVA with Bonferroni posttest. Error bars indicate the SEM throughout the figure. (C) Proteinaceous casts were scored semi-quantitatively based on histopathologic analysis of H&E-stained kidney sections. Significance bars indicate $p < 0.05$ (one-way ANOVA with Bonferroni posttest).

FIGURE 4. Significant reduction in germinal centers and plasma cells by PF-06250112. (A) The mean percentage of B220+ B cells bearing a germinal center phenotype (gated; Fas+ GL7+) is shown for the indicated treatments. $n = 6$. (B) Dot plots show the mean percentage ($n = 6$) of splenic plasma cells present in the various treatment groups. Plots are gated on “Live” singlet CD3-CD19+ splenocytes. (C) Graph shows the absolute number of germinal center B cells present in spleens obtained from the treatment groups indicated. Symbols indicate values obtained from individual animals, bars indicate the average, and error bars indicate the SEM throughout the figure. (D) Graph shows the absolute number of splenic plasma cells. **$p < 0.01$, ***$p < 0.001$, one-way ANOVA with Bonferroni posttest.
ment by quantitating the numbers of B cells present in spleen and circulation. Vehicle-treated NZBxW_F1 mice exhibited highly variable total numbers of splenic B cells with a mean of $\sim 45 \times 10^6$ per spleen and a 95% confidence interval within $+/-$ 27 $\times 10^6$ (Table I). Treatment with 3 mg/kg PF-06250112 resulted in a nonsignificant trend toward a 4-fold reduction in B cell numbers (Table I). Treatment with 10 or 30 mg/kg PF-06250112 significantly reduced total splenic B cell numbers by nearly 10-fold (Table I). Marginal zone, B1, and follicular B cell numbers were reduced by treatment with PF-06250112 with an approximate 20-, 10-, and 5-fold reduction for B1, and follicular B cell numbers, respectively, with a trend toward reduced B cells was observed at the lowest doses of PF-06250112 and was significant at the 30 mg/kg dose (Table I).

**PF-06250112 prevents the development of Ab-induced glomerulonephritis**

We next sought to determine whether treatment with a BTK inhibitor could limit renal disease development in an Fc-dependent, Ab-induced model of glomerulonephritis (8). Disease induction was induced by passive transfer of two Abs targeted to GBM. A rabbit-derived anti-murine GBM polyclonal Ab was injected 24 h before administration of a murine anti-rabbit IgG2a mAb to induce disease. Administration of this combination of Abs results in a rapid onset of severe proteinuria and is associated with deposition of rabbit Ig, murine Ig, and complement C3 within glomeruli (8). Mice treated with PF-06250112 exhibited a dose-dependent decrease in proteinuria levels (Fig. 7A, 7B). Disease development was inhibited by the PF-06250112 even in the context of glomerular deposition of Ab and C3, indicating that the compound did not impact deposition or clearance of GBM-targeted IgGs (Fig. 7C–E). Thus, these results demonstrate that BTK inhibition can limit the development of IC-driven glomerular pathology.

**Discussion**

In this article, we report a novel potent BTK inhibitor that exhibits high selectivity for BTK. Moreover, we demonstrate efficacy with this compound in two murine models of glomerulonephritis. PF-06250112 prevented the development of proteinuria in both a spontaneous model of SLE and an effector phase–restricted model mediated by administration of anti-GBM Abs. In NZBxW_F1 mice, treatment with PF-06250112 significantly reduced spontaneous model of SLE and an effector phase–restricted model mediated by administration of anti-GBM Abs. In NZBxW_F1 mice, treatment with PF-06250112 prevented the development of glomerulonephritis in the Ab-driven, anti-GBM–induced nephritis model. Collectively, these data highlight the therapeutic benefit of treatment with a BTK inhibitor and the ability of BTK inhibition to modulate multiple key pathways in the development of glomerulonephritis.

Disease development in NZBxW_F1 mice is dependent on activation of autoreactive B cells that produce autoantibodies, which drive glomerulonephritis and renal failure. In NZBxW_F1, idd mice, which lack functional BTK expression, renal pathology does not develop (28). Similarly, we find that administration of a BTK inhibitor to NZBxW_F1 mice with established titers of autoantibodies...
prevents the development of severe proteinuria. Congenital deficiency in BTK severely impairs normal B cell development in both mice and humans (10). Thus, the failure to develop disease in NZBxW_F1.xid mice may result from skewed B cell repertoire formation (28). Long-term treatment of NZBxW_F1 mice with high doses of a BTK inhibitor recapitulated the reduced numbers of mature B cells observed in xid mice, and the associated reduction in activated B cells may result from a corresponding decrease in Ag-specific precursors. Notably, in mice treated with 3 mg/kg PF-06250112, the absolute numbers of mature B cells were not significantly reduced, whereas splenic germinal center B cell numbers were decreased by 7-fold. These results suggest that treatment with a BTK inhibitor can partially impact disease development by limiting entry of B cells into the germinal response pathway, which may ultimately reduce the quality of autoantibodies produced. Interestingly, transgenic expression of a BTK hypomorph in an xid background that exhibits 25% of normal expression levels results in the generation of normal numbers of mature B cells but impaired B cell Ab production after immunization (29). Conversely, transgenic overexpression of BTK results in spontaneous germinal center formation, autoantibody production, and SLE-like disease (30). Thus, these results collectively highlight the critical importance of BTK in regulating B cell activation and tolerance. Future studies will determine the extent of BTK inhibition required to affect these parameters, as these results will have important implications for therapeutic dosing of a BTK inhibitor in the clinic.

FcγR signaling is required for the development of glomerulonephritis in NZBxW_F1 mice (7). FcγR is a coreceptor for FcγRI and FcγRIII that provides intracellular signaling capabilities through its intracellular ITAM motif, and cross-linking of FcγRIII induces BTK-dependent cytokine production in macrophages (11, 17). We demonstrate that PF-06250112 can directly inhibit BTK-dependent FcR activation in a human whole-blood IgE-mediated degranulation assay. Importantly, NZBxW_F1.FcγR−/− mice are protected from developing proteinuria; however, these mice nevertheless develop high titers of anti-dsDNA autoantibodies and exhibit abundant IgG and complement deposition in their kidneys (7). These results highlight the importance of FcγR signaling as a distal component of disease pathogenesis in NZBxW_F1 mice. In this regard, it is particularly noteworthy that treatment with 3 mg/kg PF-06250112 did not significantly limit accumulation of anti-dsDNA Abs relative to vehicle-treated mice; however, strikingly, none of these animals developed proteinuria during the study course. Moreover, IgG and complement deposition in kidney were reduced, but not completely ablated. This result suggests that the various BTK-dependent cellular functions identified to date may exhibit unique sensitivities to inhibition, and that the efficacy observed particularly at the lowest doses of PF-06250112 may be derived from prominent effects on FcγR inhibition. Future

**Table I. Impact of PF-06250112 on B cell subsets in spleen and blood**

<table>
<thead>
<tr>
<th>Subset</th>
<th>Treatment</th>
<th>Averagea</th>
<th>SEM</th>
<th>95% CI</th>
<th>Averagea</th>
<th>SEM</th>
<th>95% CI</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B220</td>
<td>Vehicle</td>
<td>44.73</td>
<td>10.58</td>
<td>27.21</td>
<td>1.47</td>
<td>0.33</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>3 mg/kg</td>
<td>11.57</td>
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<td>5.81</td>
<td>0.525</td>
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<tr>
<td></td>
<td>10 mg/kg</td>
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<td>0.855</td>
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<td>3.31</td>
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<td>Follicular</td>
<td>Vehicle</td>
<td>16.22</td>
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<td>0.21</td>
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<td>5.12</td>
<td>1.12</td>
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<td>1.78</td>
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<td>30 mg/kg</td>
<td>1.37</td>
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<td>0.106</td>
<td>0.01</td>
<td>0.03</td>
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<tr>
<td>Marginal zone</td>
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<td>9.42</td>
<td>4.62</td>
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<tr>
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<td>2.68</td>
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<tr>
<td></td>
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<td>1.21</td>
<td>0.09</td>
<td>0.22</td>
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<td>N/A</td>
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<tr>
<td></td>
<td>30 mg/kg</td>
<td>0.88</td>
<td>0.12</td>
<td>0.28</td>
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<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>B1</td>
<td>Vehicle</td>
<td>11.74</td>
<td>2.84</td>
<td>7.30</td>
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<td>N/A</td>
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</tr>
<tr>
<td></td>
<td>3 mg/kg</td>
<td>2.02</td>
<td>0.90</td>
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<td>10 mg/kg</td>
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<td>0.06</td>
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<tr>
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<td>30 mg/kg</td>
<td>0.51</td>
<td>0.11</td>
<td>0.26</td>
<td>ND</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*Absolute cell counts (×10⁶).

**Cell counts (cells [×10³]/µL).

Dashes denote p > 0.05.

*p < 0.05, **p < 0.01, ***p < 0.001, ANOVA.

N/A, Not applicable.

**FIGURE 7.** PF-06250112 inhibits the development of Ab-induced glomerulonephritis. (A) Proteinuria scores were obtained from anti-GBM Ab-injected mice that were treated with vehicle or the indicated doses of PF-06250112. Symbols indicate the mean values obtained from n = 5 mice, and error bars indicate the SEM. (B) Proteinuria scores obtained 24 h after induction of glomerulonephritis are shown for mice treated with vehicle or PF-06250112. *p < 0.05, **p < 0.01, Kruskal–Wallis with Dunn’s post-test throughout the figure. Immunofluorescence images show representative images of glomeruli from mice treated as indicated and stained for (C) rabbit IgG, (D) murine IgG, or (E) C3 deposition. Scale bars, 50 μm.
studies will explore this possibility further. Expression of FcRγ by hematopoietic cells is required for the development of proteinuria in NZBxW_F1 mice; however, the particular leukocyte population(s) that contributes to disease pathogenesis remains unclear (6). As nephritis worsens in patients and NZBxW_F1 mice, the renal interstitium becomes infiltrated with activated macrophage and dendritic cell populations that produce chemokines and inflammatory cytokines, and it is tempting to speculate that these cell types may serve as potential FcR-activated target populations for BTK-mediated therapy in SLE (31–34). To better understand the role of BTK in inhibiting FcR-dependent kidney pathology, we established a passive transfer model of anti-GM Ab–induced glomerulonephritis (8). Multiple studies have shown that Ab-mediated glomerulonephritis is mediated by FcR-expressing hematopoietic cells that include macrophages and neutrophils (35, 36). Treatment with PF-06250112 significantly reduced disease development without impacting anti-GM Ab or complement deposition in the kidney. TNF-α production by activated PMNs in anti-GM-induced glomerulonephritis is correlated with disease development and contributes to disease pathogenesis in a rat-based anti-GM model (37). Thus, BTK inhibition may limit the development of proteinuria in anti-GM–induced nephritis by preventing cytokine production by macrophages, as well as neutrophils.

Treatment of lupus-prone MRL/lpr and SLE1/SLE1.SLE3 congenic mice with a BTK inhibitor only modestly impacted anti-dsDNA autoantibody titers (23). The extent to which BTK was inhibited in MRL/lpr mice and our studies is currently unknown; thus, variations in the degree of BTK inhibition may be partially responsible for the observed differences in efficacy. In addition, the ability of BTK inhibition to limit activation of cellular pathways that are tied to disease pathogenesis in MRL/lpr was not reported. It is important to note that glomerulonephritis develops via unique mechanisms in each strain. MRL/lpr mice develop renal pathology in a B cell–dependent manner that does not require secretion of autoantibody, and B cells are thought to promote disease via their Ag-presenting capabilities and cytokine production (38). Moreover, and in direct contrast with NZBxW_F1 mice, glomerulonephritis develops in MRL/lpr mice in an FcRγ-independent manner (39). Given the highly heterogeneous nature of SLE, each of these models may be reflective of mechanistic pathways operating in subsets of SLE patients. Thus, understanding the particular cell types in which BTK is activated in both mice and patients may define patient populations that respond robustly to BTK inhibition therapy.

A similar report detailing the impact of treatment of NZBxW_F1 mice with a BTK inhibitor was published while this manuscript was in review (40). Consistent with our findings, Mina-Osorio (40) demonstrate that treatment of NZBxW_F1 mice with RN486, which is a reversible selective inhibitor of BTK, prevents the development of severe proteinuria and renal pathology. In addition, splenetic plasma cell numbers were significantly reduced and the accumulation of IgG anti-dsDNA autoantibodies was halted upon initiation of treatment with RN486 (40). The two compounds differ, however, in their respective impact on total splenic IgG ASCs and B cell numbers. PF-06250112 profoundly reduced both of these populations, whereas RN486 had more modest effects. In this regard, it is noteworthy that PF-06250112 and RN486 exhibit distinct selectivity windows for TEC kinase (2× and 206×, respectively) (41). TEC is a nonreceptor tyrosine kinase that is expressed by both B and T cells. TEC-deficient mice exhibit normal lymphocyte development and activation (42). Importantly, however, BTK and TEC double knockout mice exhibit a dramatic reduction in total B cell numbers that is greater than that observed in mice solely deficient in BTK, indicating that TEC can compensate for the loss of BTK in mice (42). Thus, the profound reduction in total B cell numbers observed after treatment with PF-06250112 might reflect inhibition of a TEC-mediated compensatory pathway. The distinct effects of PF-06250112 and RN486 on anti-dsDNA versus total IgG ASCs is also noteworthy because these results suggest that TEC-mediated signaling may contribute to distinct classes of autoantibody production in NZBxW_F1 mice.

Activated CD4+ T cells accumulate in NZBxW_F1 mice and support autoreactive B cell activation and maturation (27). Surprisingly, treatment with PF-06250112 significantly reduced the numbers of activated CD4+ T cells, whereas naive CD4+ T cell numbers remained affected. In vitro treatment of CD3/CD28-stimulated human or murine T cells with PF-06250112 suppressed T cell proliferation only modestly at high micromolar concentrations of inhibitor. T cells express the Tec family kinases IL-2–inducible T cell kinase, TXK tyrosine kinase, and TEC, and based on enzyme activity assays, we show that PF-06250112 exhibits a 10,000–, 420-, and 2.4-fold selectivity for BTK over each of these members, respectively (43). Given the selectivity window for TEC and TXK in particular, it is noteworthy that TEC and TXK knockout mice exhibit normal T cell development, as well as activation in vitro (44). Thus, the reduction in activated CD4+ T cells in vivo likely results from an indirect effect of BTK inhibition on other cell types. Notably, NZBxW_F1 mice, which have received B cell depletion therapy mediated by an anti-CD20–depleting Ab, show a similar reduction in activated CD4+ T cells (45). These results indicate that B cells are required for the stimulation or maintenance of activated CD4+ T cells in NZBxW_F1 mice. BTK has also been suggested to regulate Ag processing and presentation after BCR cross-linking, which may limit the accumulation of activated CD4+ T cells (46). Also, the reduction in total B cell numbers after treatment with high-dose PF-06250112 may physically limit the availability of cognate peptides to Ag-specific T cell clones. Importantly, activation of CD4+ T cells may require other BTK-expressing APC populations, which include dendritic cells and macrophages. Notably, both FcR and TLR signaling pathways have been identified that use BTK as a downstream signaling component for their activation (17, 47).

BTK plays a critical role in Ab production, particularly for IgM and IgG3 isotypes. Notably, NZBxW_F1 mice develop hypergammaglobulinemia that worsens with age. We observed that levels of all IgG isotypes and IgM were increased in vehicle-treated animals by a factor of 3- to 6-fold at the end of our studies compared with their pretreatment levels. Although treatment with PF-06250112 for 12 wk was associated with a profound reduction in mature and activated B cell populations, total IgG1, IgG2a, and IgG2b levels were not significantly impacted. This is consistent with the observation that long-lived plasma cells do not express BTK (9). Thus, treatment with a BTK inhibitor is unlikely to significantly decrease autoantibody-secreting long-lived plasma cells, although efficacy may be derived from inhibition of IC FcR-driven activation. FcR-dependent phagocytosis of ICs has been reported to require BTK, and it will therefore be important to understand in future studies how the levels of circulating ICs are impacted by treatment with a BTK inhibitor (48, 49). In contrast with the aforementioned Ig isotypes, IgG3 levels were significantly reduced compared with baseline levels after treatment of NZBxW_F1 mice with PF-06250112. This observation is consistent with the significant reduction in IgG3 titers observed in iid mice and the critical role that BTK plays in supporting T-independent Ab responses (19, 50).

In conclusion, we demonstrate that treatment with a potent, highly selective BTK inhibitor profoundly limits disease patho-
References
5. Bergtold, A., A. Gavhane, V. D’Agati, M. Madaio, and R. Clynes. 2006. FcR- 

Disclosures
All authors are either current or former Pfizer employees who contributed to the study design, data generation, and/or interpretation.

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ration of histology samples; M. Hegen and C. Wrocklage for hematology support; D. Carrar for collection of urinalysis end points; A. Widom for external collaborative support; C. Ambler, L. Deschenes, and J. Davis for formulation and compound support; and B. Rago for supporting the assessment of pharmacokinetic end points.

BTK INHIBITION PREVENTS GLOMERULONEPHRITIS

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Servier

Downloaded from www.jimmunol.org on May 13, 2022.


Supplementary Table 1.
Enzyme based selectivity assessment

<table>
<thead>
<tr>
<th>Kinase</th>
<th>IC50 (nM)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>BMX*</td>
<td>0.91</td>
<td>1.84</td>
</tr>
<tr>
<td>Tec*</td>
<td>1.2</td>
<td>2.4</td>
</tr>
<tr>
<td>RLK*</td>
<td>210</td>
<td>420</td>
</tr>
<tr>
<td>ITK*</td>
<td>5000</td>
<td>10000</td>
</tr>
<tr>
<td>ERBB4*</td>
<td>104</td>
<td>208</td>
</tr>
<tr>
<td>ERBB2*</td>
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</tr>
<tr>
<td>BLK*</td>
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<td>1076</td>
</tr>
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<td>EGFH*</td>
<td>6819</td>
<td>13620</td>
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<tr>
<td>JAK3*</td>
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</tr>
<tr>
<td>Lyn</td>
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<td>2180</td>
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<tr>
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</tr>
<tr>
<td>Lck</td>
<td>4170</td>
<td>8340</td>
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</table>

* - designates kinases containing a conserved cysteine residue in the active site
† - indicates measurements were obtained by Lantha screen assay.
§ - results were obtained by Invitrogen SelectScreen® Kinase profiling.
Supplementary Table 2. Impact of PF-06250112 on serum Ig titers

<table>
<thead>
<tr>
<th>Isotype</th>
<th>Treatment</th>
<th>Mean*</th>
<th>SEM</th>
<th>95% CI</th>
<th>Fold-change†</th>
<th>Significance‡</th>
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<tr>
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<tr>
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<td>353</td>
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<td>34.1</td>
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<td>1.9</td>
<td>-</td>
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* Mean terminal Ig titers. †Fold change measurements compare titers at necropsy relative to those obtained just prior to study start. Pretreatment titers were similar across all treatment groups. ‡Significance is indicated relative to vehicle treated animals. *p < 0.05; ** p < 0.01; ANOVA.
Supplementary Figure 1. Representative flow cytometry plots. A. Histograms show CD69 levels on CD19+ SSClo cells following anti-BCR stimulation under the conditions listed. B. Dot plots show the gating strategy used to identify marginal zone, follicular and B1 B cells.
Supplementary Figure 2. Pharmacokinetic properties of PF-06250112 in NZBxW_F1 mice. 

A. 26 week old NZBxW_F1 mice were administered a single dose of either 1 mg/kg (black squares) or 10 mg/kg (grey circles) of PF-06250112. Graph shows exposures obtained at various time points post-dosing. \( N \geq 2 \) mice per time point. Error bars indicate the standard error of the mean. 

B. Table shows the pharmacokinetic profile of PF-06250112 at the indicated doses in NZBxW_F1 mice. * indicates extrapolated value. 

C. Graph shows the plasma concentrations of PF-112 measured bi-weekly from day 14 to 84 on study. \( N \geq 8 \) mice per time point.