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Inhibition of Btk with CC-292 Provides Early Pharmacodynamic Assessment of Activity in Mice and Humans[S]

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ABSTRACT

Targeted therapies that suppress B cell receptor (BCR) signaling have emerged as promising agents in autoimmune disease and B cell malignancies. Bruton's tyrosine kinase (Btk) plays a crucial role in B cell development and activation through the BCR signaling pathway and represents a new target for diseases characterized by inappropriate B cell activity. *N*-(3-(5-fluoro-2-(4-(2-methoxyethoxy)phenylamino)pyrimidin-4-ylamino)phenyl)acrylamide (CC-292) is a highly selective, covalent Btk inhibitor and a sensitive and quantitative assay that measures CC-292-Btk engagement has been developed. This translational pharmacodynamic assay has accompanied CC-292 through each step of drug discovery and development. These studies demonstrate the quantity of Btk bound by CC-292 correlates with the efficacy of CC-292 in vitro and in the collagen-induced arthritis model of autoimmune disease. Recently, CC-292 has entered human clinical trials with a trial design that has provided rapid insight into safety, pharmacokinetics, and pharmacodynamics. This first-in-human healthy volunteer trial has demonstrated that a single oral dose of 2 mg/kg CC-292 consistently engaged all circulating Btk protein and provides the basis for rational dose selection in future clinical trials. This targeted covalent drug design approach has enabled the discovery and early clinical development of CC-292 and has provided support for Btk as a valuable drug target for B-cell mediated disorders.

Introduction

Bruton's tyrosine kinase (Btk) is a kinase expressed exclusively in B cells and myeloid cells and has a well characterized, vital role in B cells highlighted by the human primary immune deficiency disease, X-linked agammaglobulinemia (XLA), which results from mutation in the Btk gene (Smith et al., 1998). As a result of incomplete B cell differentiation, XLA patients have a near complete absence of mature B cells in the peripheral blood (Campana et al., 1990) and cannot produce immunoglobulins (Conley, 1985; Nonoyama et al., 1998). The human XLA phenotype is recapitulated, although less severely, in Btk knock-out mice (Khan et al., 1995) and in *xid* mice, which have a naturally occurring Btk mutation (Rawlings et al., 1993).

Specifically, Btk plays an essential role in the B cell receptor (BCR) signaling pathway. Antigen binding to the BCR results in B cell receptor oligomerization, Syk and Lyn kinase activation (Gauld et al., 2002), followed by Btk kinase activation (Park et al., 1996; Rawlings et al., 1996; Baba et al., 2001). Once activated, Btk forms a signaling complex with proteins such as BLNK, Lyn, and Syk and phosphorylates phospholipase C (PLC)γ2 (Baba et al., 2001; Tsukada et al., 2001). This leads to downstream release of intracellular Ca^{2+} stores and propagation of the BCR signaling pathway through extracellular signal-regulated kinase and nuclear factor-κB signaling, ultimately resulting in transcriptional changes to foster B cell survival, proliferation, and/or differentiation (Baba et al., 2001; Maas and Hendriks, 2001; Mohamed et al., 2009).

While BCR signaling is essential in the normal development and function of B cells, several pathologies have been attributed to dysregulated BCR activity. These include diseases of autoreactivity, such as that observed in lupus, multiple sclerosis, and rheumatoid arthritis, in which B cells inappropriately break self-tolerance to produce antibodies contributing to autoimmune disease (Edwards and Cambridge, 2005, 2006; Teng et al., 2007). BCR signaling also contributes to several B cell malignancies, such as chronic lymphocytic leukemia (CLL) (Chen et al., 2005; Hoellenriegel et al., 2011; Stevenson et al., 2011), mantle cell lymphoma, and subsets of diffuse large B cell leukemia (Chen et al., 2008; Lenz et al., 2008; Baran-Marszak et al., 2010; Davis et al., 2010; Suljagic et al., 2010; Pighi et al.,

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ABBREVIATIONS: BCR, B cell receptor; BSA, bovine serum albumin; Btk, Bruton's tyrosine kinase; CC-292, *N*-(3-(5-fluoro-2-(4-(2-methoxyethoxy)phenylamino)pyrimidin-4-ylamino)phenyl)acrylamide; CIA, collagen-induced arthritis; CLL, chronic lymphocytic leukemia; CNX-500, N^1-(3-(3-(4-(3-acrylamidophenylamino)-5-methylpyrimidin-2-ylamino)phenoxy)propyl)-N^5-(15-oxo-19-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl)-4,7,10-trioxa-14-azanonadecyl)glutaramide; CST, Cell Signaling Technology; ELISA, enzyme-linked immunosorbet assay; PBS, phosphate-buffered saline; PD, pharmacodynamic; PK, pharmacokinetic; PLC, phospholipase C; XLA, X-linked agammaglobulinemia.

2011). However, until recently, therapies that target the B cell have resulted in depletion of the B cell repertoire, while therapeutic strategies that reduce BCR activity are relatively new for treatment of these diseases.

Promising recent clinical data generated by inhibition of distinct BCR signaling components, including Syk, phosphatidylinositide 3-kinase δ, and Btk with fostamatinib, idelalisib (also known as GS-1101 or CAL-101), and ibrutinib (PCI-32765), respectively, have provided great excitement for this approach. Inhibition of Syk with fostamatinib has demonstrated efficacy in human clinical trials in rheumatoid arthritis as well as in B cell malignancies dependent on BCR signaling such as CLL (Braselmann et al., 2006; Chen et al., 2008; Podolanczuk et al., 2009; Friedberg et al., 2010; Genovese et al., 2011). Similarly, inhibition of phosphatidylinositide 3-kinase δ with GS-1101 has also shown promising results in CLL (Herman et al., 2010; Hoellenriegel et al., 2011; Lannutti et al., 2011). Btk, downstream of Syk in the BCR signaling pathway, also represents an attractive drug target in diseases characterized by aberrant B cell activity. Moreover, owing to its highly restricted expression pattern in B cells and myeloid cells, Btk provides an opportunity for selective therapeutic targeting. Preclinically, small molecule inhibition of Btk with CGI1746 and ibrutinib demonstrated therapeutic activity in several models of autoimmune disease (Honigberg et al., 2010; Chang et al., 2011; Di Paolo et al., 2011). Ibrutinib has shown promising results in early clinical development for the treatment of B cell malignancies (Harrison, 2012; Advani et al., 2013) and is currently in phase III trials in CLL, providing evidence that Btk represents a viable and efficacious therapeutic target.

We describe our work on *N*-(3-(5-fluoro-2-(4-(2-methoxyethoxy)phenylamino)pyrimidin-4-ylamino)phenyl)acrylamide (CC-292), which is a potent, highly selective, covalent inhibitor of Btk that inhibits BCR signaling and has efficacy in a rheumatoid arthritis disease model. We also describe a pharmacodynamic (PD) assay that has been implemented throughout all stages of preclinical development to measure activity of CC-292 and correlate Btk inhibition with functional outcome both in vitro and in vivo. Finally, we report the substantial oral exposure to CC-292 in humans and use this PD assay to unequivocally and quantitatively demonstrate complete Btk engagement in a first-in-human setting. This work represents the first report of a selective Btk inhibitor appropriate for use in a human clinical setting of autoimmune disease and uses a powerful translational approach to confirm on-target activity in human B cells.

Materials and Methods

B-Lymphocyte Isolation for In Vitro Signaling, Proliferation, and Activation. Human naïve, primary B cells (CD19+, IgD+) were isolated from anticoagulated whole blood by density centrifugation through Histopaque-1077 and peripheral blood mononuclear cell (PBMC) isolation. PBMCs were subject to red blood cell lysis using Red Blood Cell Lysis Buffer (Boston BioProducts, Ashland, MA) followed by incubation with MACS reagent (130-091-150) and negative selection over a MACS column to obtain naïve primary B cells with >85% purity.

Immunoblot Analysis. Cells were incubated in serum-free RPMI media for 1–1.5 hours. Isolated human B cells were incubated with CC-292 at final concentrations of 0.001, 0.01, 0.1, and 1 μM. Resting cells were incubated in the presence of compound for 1 hour at 37°C. Following incubation, cells were centrifuged and resuspended in 100 μl of serum-free RPMI and BCR was stimulated with addition of 5 μg/ml α-human IgM. Samples were centrifuged, washed in phosphate-buffered saline (PBS), and lysed in 100 μl of Cell Extraction Buffer [cat. no. FNN0011; Life Technologies (Invitrogen), Carlsbad, CA] plus 1:10 (v/v) PhosSTOP Phosphatase Inhibitor (Roche, Basel, Switzerland) and 1:10 (v/v) Complete Protease Inhibitor (cat. no. 11836145001; Roche). Antibodies used for immunoblot analysis include P-PLCγ2 [cat. no. 3872; Cell Signaling Technology, Beverly, MA (CST)], PLCγ2 (3871; CST), Syk (2712; CST), P-Syk (2710; CST), Btk (cat. no. 611116; BD Biosciences, Franklin Lakes, NJ), P-Btk (cat. no. 2207-1; Epitomics, Berlingame, CA), and Tubulin (cat. no. T6199; Sigma-Aldrich, St. Louis, MO). Membranes were scanned on a Li-Cor Odyssey scanner using infrared fluorescence detection (Li-Cor Biosciences, Lincoln, NB).

B-Lymphocyte Proliferation (^{3}H-Thymidine Incorporation). A suspension of resting purified naïve human B cells isolated by negative selection (MACS reagent 130-091-150) in RPMI was prepared at 0.4–0.5 × 10^6 cells/ml. Cells were mixed together with α-human IgM (final concentration of 5 μg/ml in each well) and vehicle (dimethyl sulfoxide) or CC-292 (final concentrations of 0.01, 0.1, 1.0, 10.0, 100.0, or 1000 nM per well) and seeded in a 96-well plate. Cells were incubated for 56 hours in a humidified incubator maintained at 37°C and 5% CO_2. ^{3}H-Thymidine was added (final concentration of 1 μCi in each well) and cells were incubated overnight, harvested, and measured for ^{3}H incorporation. Experiments were performed in triplicate.

Btk Target Site Occupancy Enzyme-Linked Immunosorbent Assay. An enzyme-linked immunosorbent assay (ELISA) method for the detection of free uninhibited Btk in mouse, rat, dog, monkey, and human lysates was developed at Celgene Avilomics Research, and a validation of this method in human B cell lysate was performed by a federal Certified Laboratories Improvement Amendments-certified laboratory (Cambridge Biomedical Laboratories, Boston, MA) The parameters that were assessed included: accuracy, linearity, dilution, precision (intra- and interassay), stability, reference range, freeze-thaw cycles, reportable range, specificity, sensitivity, and carryover. All specifications for linearity, precision (intra- and interassay), accuracy, and carryover defined in the validation protocol were met. Samples were stable at –80°C for 5 weeks and the reportable range of the Btk ELISA was 12–12,800 pg of free Btk. Cell lysates or spleen homogenates were incubated with N^1-(3-(3-(4-(3-acrylamidophenylamino)-5-methylpyrimidin-2-ylamino)phenoxy)propyl)-N^5-(15-oxo-19-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl)-4,7,10-trioxa-14-azanonadecyl)glutaramide (CNX-500) (Celgene Avilomics Research; final concentration 1 μM) in a PBS, 0.05% Tween-20, 1% bovine serum albumin (BSA) solution for 1 hour at room temperature. Standards and samples were transferred to a streptavidin-coated 96-well ELISA plate and mixed while shaking for 1 hour at room temperature. The α-Btk antibody (BD 611116, 1:1000 dilution in PBS + 0.05% Tween-20 + 0.5% BSA) was then incubated for 1 hour at room temperature. After wash, goat anti-mouse horseradish peroxidase (1:5000 dilution in PBS + 0.05% Tween-20 + 0.5% BSA) was added and incubated for 1 hour at room temperature. The ELISA was developed with addition of tetramethyl benzidine followed by Stop Solution (CST) and read at optical density 450 nm. The standard curve (11.7–3000 pg/μl) was generated with human full-length recombinant Btk protein and plotted using a 4-parameter curve fit in Gen5 software. Uninhibited Btk detected from samples was normalized to μg total protein as determined by BCA protein analysis (cat. no. 23225; Pierce, Rockford, IL).

Spleen Homogenization. Spleens were harvested from mice, frozen immediately in liquid nitrogen, and stored at –80°C. To generate spleen lysates, each spleen was sliced in half and lysed using a Precellys 24 Bead Homogenizer in 500 μl of Bio-Rad Bio-Plex Lysis Buffer plus protease inhibitors (Bio-Rad, Hercules, CA). Supernatant

Collagen-Induced Arthritis Model. Experiments were carried out at Bolder Biopath, Boulder, CO. All experiments were carried out in compliance with regulations of the Institutional Animal Care and Use Committee and were conducted in accordance with principles and procedures dictated by the highest standards of humane animal care. Dba1 mice were injected at the base of the tail with 150 μl of Freund's Complete Adjuvant (Sigma-Aldrich) containing bovine type II collagen (Elastin Products, Owensville, MO) (2 mg/ml) on day 0 and again on day 21. On study days 25–27, onset of arthritis occurred, and mice were randomized into treatment groups (10 per treatment group, four per group for normal). Randomization into each group was done after swelling was obviously established in at least one paw, and attempts were made to assure approximately equal mean scores across the groups at the time of enrollment. Treatment was initiated after enrollment. Treatment continued daily (QD at 24-hour intervals) through arthritis day 14. Clinical scores were assessed for each of the paws on study arthritis days 1–15 using the following scoring system: 0 = normal, 1 = one hind or fore paw joint affected or minimal diffuse erythema and swelling, 2 = two hind or fore paw joints affected or mild diffuse erythema and swelling, 3 = 3 hind or fore paw joints affected or moderate diffuse erythema and swelling, 4 = marked diffuse erythema and swelling or 4 digit joints affected, 5 = severe diffuse erythema and severe swelling entire paw, unable to flex digits. Spleens and plasma were harvested 2 or 24 hours after the last dose of CC-292 on arthritis day 14 and paws were removed and fixed in formalin for histopathological analysis.

Clinical Study. A double-blind, placebo-controlled, ascending single-dose, randomized study in normal healthy human volunteers was conducted at a single clinical research unit in accordance with Declaration of Helsinki principles. Informed consent statements were obtained from all subjects prior to inclusion in the study. Subjects were admitted to the unit 1 day before dosing and discharged 96 hours after dosing. Six subjects were administered a single oral dose of 2 mg/kg CC-292, monitored for safety and evaluated for drug action by pharmacokinetic (PK) and PD analysis.

Isolation of Enriched B Lymphocyte Population from Human Healthy Volunteers. Human whole blood (21 ml) was collected from each subject at each time point into BD Vacutainer CPT Cell Preparation Tubes containing sodium heparin. RosetteSep Human B Cell Enrichment Cocktail (cat. no. 15024; StemCell Technologies, Vancouver, BC, Canada) was added to each CPT tube and centrifuged for 25 minutes at 1800*g* at room temperature. Isolated cells were harvested into a clean 50-ml conical tube that was pooled by subject. Each enriched B cell suspension was centrifuged at 400*g* for 15 minutes at room temperature. Cell suspensions were diluted in 1 ml of red blood cell lysis buffer for 3 minutes at room temperature. Cell pellets were lysed with 150 μl Bio-Plex lysis buffer (cat. no. 171-304012; Bio-Rad). The lysates were stored frozen at ≤–70°C until Btk target site occupancy analysis by ELISA.

Results

CC-292: A Potent, Highly Selective Btk Inhibitor. We have identified CC-292 (Fig. 1; Supplemental Fig. 1) as a potent, selective inhibitor of Btk. CC-292 was rationally designed to possess high affinity for the ATP binding pocket and to form a specific covalent bond with cysteine 481 in Btk, a poorly conserved amino acid among kinases. In biochemical assays, CC-292 is a potent inhibitor of Btk kinase activity ($IC_{50apparent} < 0.5$ nM, $k_{inact}/K_I = 7.69 \times 10^4$ $M^{-1}s^{-1}$) and is highly selective (Supplemental Tables 1 and 2; see Supplemental Methods for more information). Because biochemical kinase assays may overestimate the potency of small molecule kinase inhibitors due to high ATP concentrations found in the cellular environment, cell activity for several of these closely related kinase family members was assessed. CC-292 demonstrated a high degree of selectivity against kinases with a cysteine in a homologous position as Cys481 in Btk (epidermal growth factor receptor, Itk, Janus kinase 3; Supplemental Tables 3 and 4). Full details of the in vitro properties of CC-292 as well as confirmation of the covalent mechanism of action by mass spectrometry are shown in Supplemental Figs. 2–4 and Supplemental Tables 1–3. To demonstrate specific inhibition of Btk in cells, CC-292 was evaluated in Ramos cells, which express an intact BCR signaling pathway that is activated robustly by addition of anti-IgM. CC-292 potently inhibited Btk autophosphorylation on Tyr223 (EC_{50} = 8 nM; Fig. 2A; Supplemental Fig. 5), phosphorylation of the Btk substrate, PLCγ2, as well as activation of the downstream kinase extracellular signal-regulated kinase, all previously shown to be sensitive to Btk inhibition (Honigberg et al., 2010; Di Paolo et al., 2011). It is noteworthy that while CC-292 inhibited autophosphorylation of Btk, it had no effect on the phosphorylation of Btk on Tyr551, a site phosphorylated by Lyn and Syk and required for Btk activation (Afar et al., 1996). These data demonstrate CC-292 is selective for Btk and does not inhibit the Src-family kinases upstream of Btk in the BCR signaling pathway (Fig. 2A).

Consistent with its covalent mechanism of action, CC-292 provided prolonged inhibition of kinase activity hours after the drug was removed from cells. In contrast to reversible inhibition with the potent Btk inhibitor dasatinib (Hantschel et al., 2007), for which kinase activity had almost completely returned 6 hours after drug removal, recovery of Btk activity following a 1-hour exposure to CC-292 continued to be suppressed ~8 hours in drug-free media (Fig. 2B). This prolonged period of Btk inhibition correlated well with Btk protein turnover assayed in the presence of the protein synthesis inhibitor cyclohexamide. These experiments indicated that existing cellular Btk was degraded slowly (36% reduction of protein in 8 hours and 63% reduction at 17 hours) (Supplemental Fig. 6). Since Btk exposed to CC-292 is irreversibly bound and inhibited, the return of Btk-dependent signaling relies on the appearance of new Btk protein as a result of protein synthesis in a CC-292–free environment.

Quantitative Analysis of Btk Occupancy. The covalent mechanism of action of CC-292 has enabled design of a companion PD assay that directly quantifies covalent bonding to Btk protein after drug exposure. A probe (CNX-500) was developed consisting of a covalent Btk inhibitor chemically linked to biotin (Fig. 3A; Supplemental Fig. 7). This molecule retains inhibitory activity against Btk (IC_{50app} = 0.5 nM) as well as the ability to form a covalent bond with Btk (Supplemental Fig. 8) and has demonstrated selectivity against the structurally related kinase epidermal growth

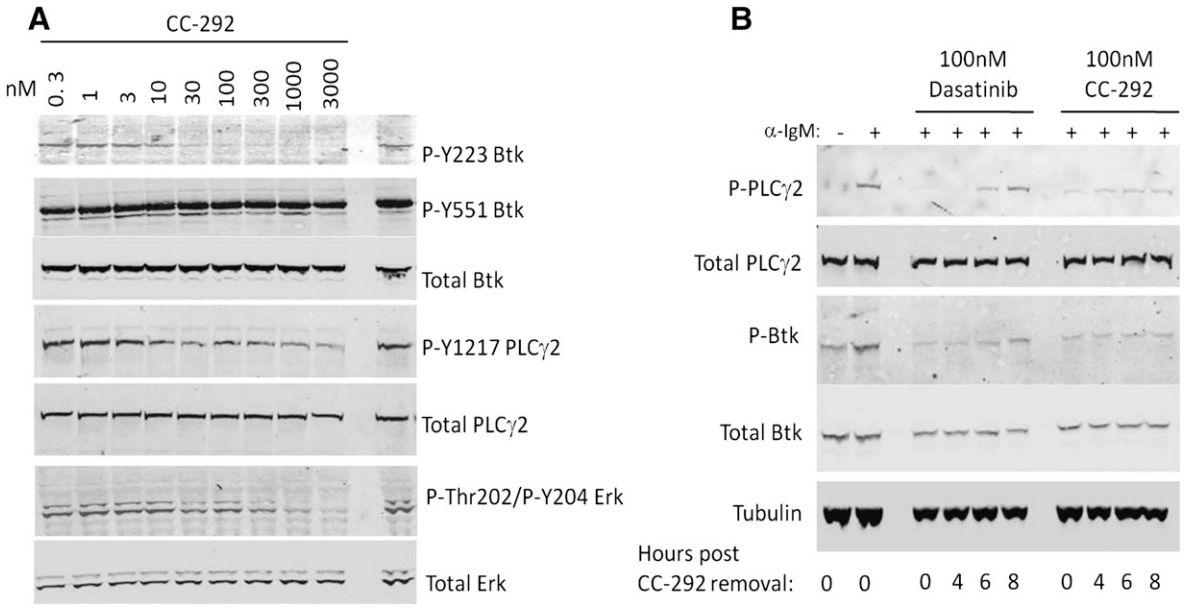

Fig. 2. CC-292 demonstrates concentration-dependent silencing of Btk activity and prolonged duration of action after α-IgM stimulation of the B cell receptor in Ramos cells. (A) Ramos cells were treated with increasing concentrations of CC-292 (0.3–3000 nM) and then stimulated with 5 μg/ml of the BCR ligand α-IgM. Btk autophosphorylation as well as Btk substrate phosphorylation (P-Y1217-PLCγ2) and downstream activation of extracellular signal-regulated kinase (Erk) were assayed by immunoblot. Quantitation of immunoblot demonstrated that CC-292 inhibits Btk autophosphorylation with EC_{50} = 8 nM (n = 4) (Supplemental Fig. 4). (B) Ramos cells were treated with compound for 1 hour. Cells were then resuspended in compound-free media and stimulated with 5 μg/ml α-IgM at 0, 4, 6, or 8 hours after compound removal. Btk substrate phosphorylation was measured by immunoblot. Btk remains inhibited up to 8 hours after treatment with the covalent modifier CC-292, whereas Btk activity returns quickly after treatment with the reversible inhibitor, dasatinib. Representative immunoblot of n = 3 experiments.

kinases including Syk (IC_{50app} > 1000 nM) and Lyn (IC_{50app} > 3500 nM). Moreover, the specificity of the Btk target occupancy ELISA derives from the use of a detection monoclonal antibody that selectively recognizes Btk immobilized on the streptavidin substrate by the covalent probe and, therefore, this assay measures only Btk bound to the covalent probe. By building a standard curve with known amounts of recombinant Btk protein bound to CNX-500, the amount of Btk in any sample can be precisely quantitated. Used in a competition assay, this probe detected free, uninhibited Btk and was excluded from interaction with Btk previously bonded by CC-292 (Fig. 3B). Results from this analysis can be reported in absolute values, such as picograms of free Btk per microgram of total protein or in relative terms by normalization to control samples not exposed to inhibitor. In Ramos cells exposed to a range of CC-292 concentrations, the amount of Btk captured by the probe was compared with untreated samples and the extent of Btk bonded was demonstrated to be proportional to CC-292 drug concentration (Supplemental Fig. 9). It is noteworthy that the degree of Btk covalently bonded by CC-292, herein referred to as Btk occupancy, correlated with inhibition of Btk kinase activity. Extensive analysis has revealed that the EC_{50} of Btk occupancy from a CC-292 dose-response in Ramos cells (EC_{50} = 6 nM) correlated directly with the cellular EC_{50} of Btk kinase inhibition with CC-292 (EC_{50} = 8 nM) (Supplemental Figs. 5 and 9). Furthermore, the concentration at which CC-292 inhibited 90% of Btk activity in Ramos cells was 35 nM while the concentration of CC-292 required for 90% occupancy of Btk was 39 nM, supporting a direct stoichiometric correlation between target occupancy and inhibition of Btk activity. This correlative relationship was

B cells ex vivo. In naïve human B cells, the kinase activity of Btk was inhibited 42% at 10 nM, a concentration that produced 37% Btk occupancy (Fig. 4A). It is noteworthy that kinase inhibition and occupancy also reflected efficacy in B cell functional assays such as B cell proliferation (EC_{50} = 3 nM; Fig. 4B) and activation as determined by inhibition of upregulation of the activation marker, CD69, in response to stimulation by anti-IgM (Supplemental Table 5). These data demonstrate a strong quantitative relationship among CC-292 concentration, extent of Btk enzyme inhibition, and level of Btk occupancy. Therefore, measurement of Btk occupancy can serve as a robust surrogate measurement of Btk kinase inhibition that correlates with inhibition of BCR signaling and its functional consequences.

As described above, once covalently bound by CC-292, an individual Btk protein is permanently silenced. Therefore, the return of activity must depend on new Btk protein synthesis. Determination of Btk protein resynthesis rates in mice in vivo was enabled by maximally inhibiting Btk with a single dose of CC-292 and then monitoring the return of Btk in spleen lysates over time with the covalent probe. Mouse spleens were collected at several time points after a single oral dose of 50 mg/kg CC-292, a dose level projected to achieve complete Btk engagement, and assayed with the covalent probe to track emergence of new Btk protein. New Btk protein was detected at low levels 8 hours after compound administration, and achieved 43% of predose Btk protein levels at 24 hours and 71% of predose levels 48 hours after drug administration (Fig. 5). It is noteworthy that PK analysis of mouse plasma from this experiment indicated circulating CC-292 was absent in five of six animals by the 8-hour time point (unpublished data). Presently, the potential contribution of active metabo

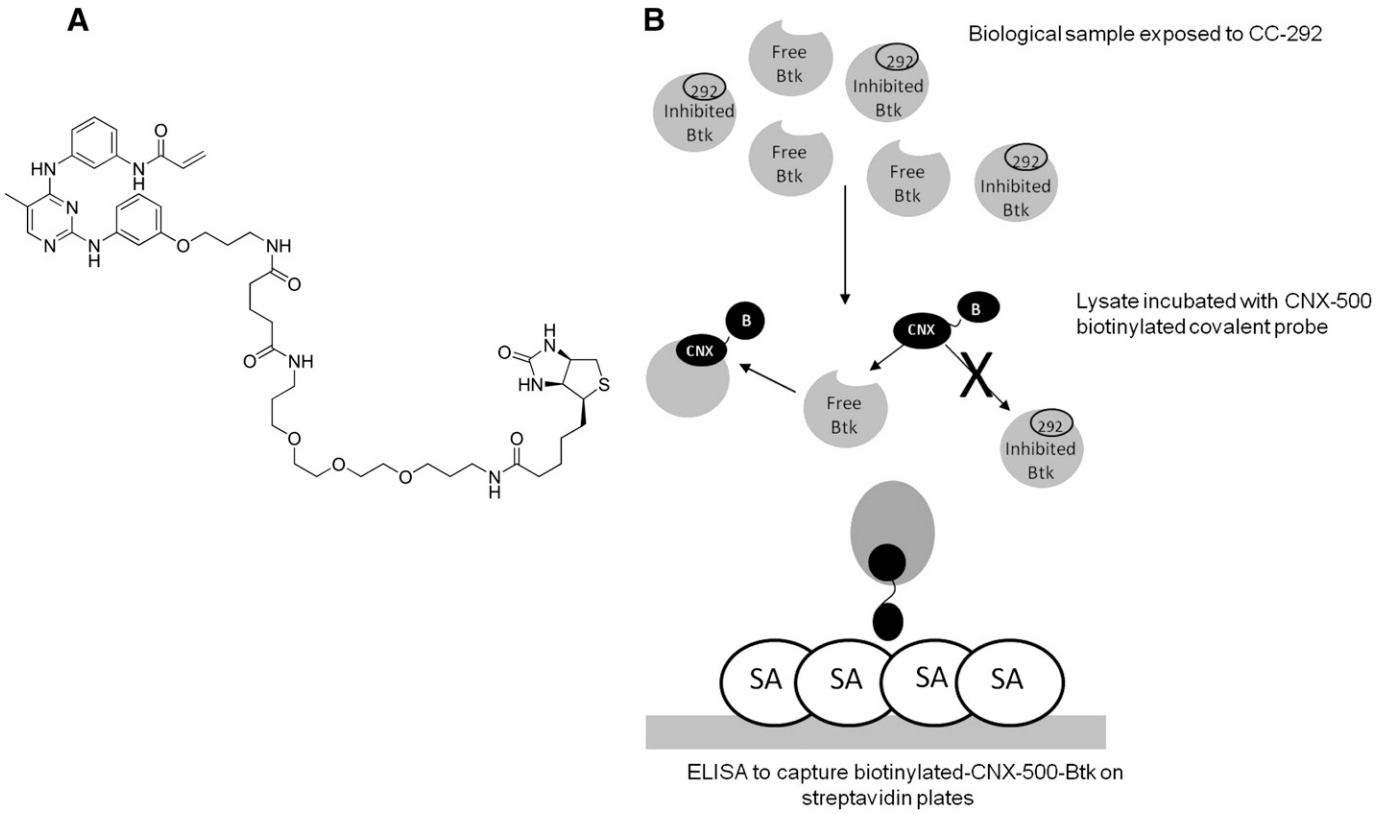

Fig. 3. Covalent probe CNX-500 allows direct assessment of Btk occupancy in vitro and in vivo. (A) Covalent probe CNX-500. (B) Covalent probe CNX-500 detects free, uninhibited Btk in lysates derived from tissue culture, animal tissues, or clinical samples. Samples treated with CC-292 are lysed and then incubated with 1 μM CNX-500. Uninhibited Btk in the lysate is captured by CNX-500 and quantitated by streptavidin (SA)-coated ELISA plate. Normalization to untreated control sample allows determination of the percentage of Btk occupancy.

determination of the extent and duration of covalent inhibition of Btk protein in mice.

Relationship of Btk Occupancy and Efficacy of CC-292 in the Collagen-Induced Arthritis Model of Arthritis. The collagen-induced arthritis (CIA) model has been shown previously to respond to both B cell modulating therapies as well as direct Btk inhibition (Pine et al., 2007; Honigberg et al., 2010; Chang et al., 2011; Di Paolo et al., 2011; Liu et al., 2011b). Oral efficacy of CC-292 in an established CIA model in mice was measured. Dose-dependent inhibition of the clinical signs of inflammatory disease was observed during the in-life portion of the model, including reduction in joint and paw swelling and visible redness of the affected paws. Reduction of clinical signs of disease was measured at 95, 85, and 50% for 30, 10, and 3 mg/kg, respectively (Fig. 6A). Moreover, all three dose levels of CC-292 prevented the loss in body weight typically associated with severity of disease observed in this model (Supplemental Fig. 10). It is noteworthy that CC-292 also demonstrated significant effects on the generation of inflammatory chemokines and cytokines in this model, including KC (mouse equivalent of interleukin-8), interleukin-6, and tumor necrosis factor α (Supplemental Table 6). The precise mechanism for this protective effect is currently under investigation but suggests direct or indirect modulation of effector cell function and may be independent of the role of Btk in B cells. To demonstrate the relationship between inhibition of inflammatory activity and direct engagement of CC-292 with Btk, dose were assayed for Btk occupancy. Occupancy in spleen lysates tracked closely with inhibition of the clinical signs of disease: 34% occupancy at 3 mg/kg at 2 hours correlated with 50% inhibition of disease, Btk occupancy of 84% was detected 2 hours after dosing with 10 (85% inhibition of disease) or 30 mg/kg (97% occupancy, 95% inhibition of disease) of CC-292. Consistent with Btk resynthesis experiments described earlier, only 19% Btk occupancy remained 24 hours after the 3-mg dose, whereas sustained occupancy of >40% at 24 hours was achieved with dose levels of 10 and 30 mg/kg. This analysis demonstrated that once-a-day dosing at the higher doses resulted in continuous CC-292–Btk engagement at levels greater than 40% and that this was sufficient for >85% inhibition of disease with therapeutic dosing of CC-292 (Fig. 6B). Morphologic and histopathologic analysis of six affected joints (four paws, two knees) demonstrated a dose-dependent protection from joint damage, including pannus formation, cartilage degradation, and bone erosion. The disease-modifying activity of CC-292 correlated with both Btk occupancy and the pronounced inhibition of the clinical inflammation characteristic of arthritis in this model (Fig. 6C). This correlation between Btk occupancy and inhibition of disease strongly suggests that selective inhibition of Btk provided the protective effect of CC-292 activity in this collagen-induced arthritis model.

Human Clinical PK-PD Relationship with CC-292. CC-292 demonstrated covalent bonding, prolonged, selective inhibition of Btk in vitro, and efficacy in preclinical models in

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