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

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Running Title: Btk Inhibition with the Covalent Inhibitor CC-292

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Nonstandard abbreviations:

BCR	B Cell Receptor
Btk	Bruton's tyrosine kinase
CIA	Collagen Induced Arthritis
CLL	Chronic Lymphocytic Leukemia
DLBCL	Diffuse Large B Cell Leukemia
ITP	Idiopathic Thrombocytopenic Purpura
MCL	Mantle Cell Lymphoma
MS	Multiple Sclerosis
PK	Pharmacokinetics
PD	Pharmacodynamics
RA	Rheumatoid Arthritis
XLA	X-linked Agammaglobulemia

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Abstract

Targeted therapies that suppress BCR signaling have emerged as promising agents in autoimmune disease and B cell malignancies. 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. 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.

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Introduction

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, 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 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 (Rawlings, et al., 1996; Park, et al., 1996; Baba, et al., 2001). Once activated, Btk forms a signaling complex with proteins such as Blnk, Lyn, and Syk and phosphorylates PLC γ 2 (Baba, et al., 2001; Tsukada, et al., 2001). This leads to downstream release of intracellular Ca²⁺ stores and propagation of the BCR signaling pathway through Erk and Nf- κ B signaling, ultimately resulting in transcriptional changes to foster B cell survival, proliferation, and/or differentiation (Maas and Hendriks, 2001; Mohamed, et al., 2009; Baba, et al., 2001).

While essential in the normal development and function of B cells, there are several pathologies that have been attributed to dysregulated BCR activity. These include diseases of autoreactivity such as that observed in lupus, MS, and RA in which B cells inappropriately break self-tolerance to produce antibodies contributing to autoimmune disease (Edwards and Cambridge, 2006; Teng, et al., 2007; Edwards and Cambridge, 2005). BCR signaling also contributes to several B cell malignancies such as CLL (Chen, et al., 2005; Hoellenriegel, et al., 2011; Stevenson, et al., 2011), MCL and subsets of DLBCL (Suljagic, et al., 2010; Chen, et al., 2008; Davis, et al., 2010; Lenz, et al., 2008; Pighi, et al., 2011; Baran-Marszak, et al., 2010). However, until recently, therapies that target the B cell have resulted in depletion of the B cell

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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, PI3K δ , and Btk with fostamatinib, idelalisib (also known as GS-1101 or CAL-101), and ibrutinib (PCI-32765), have provided great excitement for this approach. Inhibition of Syk with fostamatinib has demonstrated efficacy in human clinical trials in RA as well as in B cell malignancies dependent on BCR signaling such as CLL(Chen, et al., 2008;Friedberg, et al., 2010;Genovese, et al., 2011;Podolanczuk, et al., 2009;Braselmann, et al., 2006). Similarly, inhibition of PI3K δ with GS-1101 has also shown promising results in CLL(Lannutti, et al., 2011;Herman, et al., 2010;Hoellenriegel, 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., 2012) 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 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 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 of 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 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.

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Materials and Methods:

B Lymphocyte Isolation for in vitro signaling, proliferation, and activation: Human naïve, primary B cells (CD19+, IgD+) were isolated from anti-coagulated whole blood by density centrifugation through Histopaque®-1077 and PBMC isolation. PBMCs were subject to red blood cell lysis using Red Blood Cell Lysis Buffer (Boston Bioproducts) 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 h. Isolated human B cells were incubated with CC-292 at a final concentration of 0.001, 0.01, 0.1 and 1 μ M. Ramos cells were incubated with 0.1 nM-3 μ M CC-292. Cells were then incubated in the presence of compound for 1 h at 37°C. Following incubation, cells were centrifuged and resuspended in 100 μ L of serum-free RPMI and BCR was stimulated with addition of 5 μ g/mL α -human IgM. Samples were centrifuged, washed in PBS, and lysed in 100 μ L of Cell Extraction buffer (Invitrogen FNN0011) plus 1:10 (v:v) PhosStop® Phosphatase Inhibitor and 1:10 (v:v) Complete® Protease Inhibitor (Roche 11836145001). Antibodies used for immunoblot analysis include P-PLC γ 2 (CST 3872), PLC γ 2 (CST 3871), Syk (CST 2712), P-Syk (CST 2710), Btk (BD 611116), P-Btk (Epitomics 2207), and Tubulin (Sigma T6199). Membranes were scanned on a LiCor Odyssey Scanner using infrared fluorescence detection.

B Lymphocyte Proliferation (³H-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 X 10⁶ cells per mL. Cells were mixed together with α -human IgM (final concentration of 5 μ g/mL in each well) and vehicle (DMSO) 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 h in a humidified incubator maintained at 37°C and 5% CO₂. ³H-thymidine was added (final concentration of 1 μ Ci in each well) and

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cells were incubated overnight, harvested, and measured for ^3H incorporation. Experiments were performed in triplicate.

Btk Target Site Occupancy ELISA: An 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 CLIA Certified laboratory (Cambridge Biomedical Laboratories, Boston, MA) The parameters that were assessed included: accuracy, linearity, dilution, precision (intra and inter-assay), stability, reference range, freeze-thaw cycles, reportable range, specificity, sensitivity, and carryover. All specifications for linearity, precision (intra- and inter-assay), 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 12.5 to 12,800 pg of free Btk. Cell lysates or spleen homogenates were incubated with CNX-500 (final concentration $1\ \mu\text{M}$) in a PBS, 0.05% Tween-20, 1% BSA solution for 1 h at room temperature. Standards and samples were transferred to a streptavidin-coated 96-well ELISA plate and mixed while shaking for 1 h 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 h at room temperature. After wash, goat α -mouse-HRP (1:5000 dilution in PBS + 0.05% Tween-20 + 0.5% BSA) was added and incubated for 1 h at room temperature. The ELISA was developed with addition of tetramethyl benzidine (TMB) followed by Stop Solution and read at OD 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 (Pierce Cat. 23225).

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

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Bead Homogenizer in 500 μ L of BioRad Bio-Plex Lysis Buffer plus protease inhibitors. Supernatant was transferred to a fresh microfuge tube and stored frozen at -80°C until analysis.

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. Dbal mice were injected at the base of the tail with 150 μ L of Freund's Complete Adjuvant (Sigma) 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/treatment group, 4/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 h 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=1 hind or fore paw joint affected or minimal diffuse erythema and swelling, 2=2 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 h 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 h after dosing. Six

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subjects were administered a single oral dose of 2 mg/kg CC-292, monitored for safety and evaluated for drug action by PK and PD analysis.

Isolation of Enriched B Lymphocyte Population from Human Healthy Volunteers: 21 mL of human whole blood 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 (Stem Cell Tech. 15024) was added to each CPT tube and centrifuged for 25 minutes at 1800 x 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 x 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 μ L Bio-Rad Bio-Plex lysis buffer (Cat. #171-304012). The lysates were stored frozen at $\leq -70^{\circ}\text{C}$ until Btk target site occupancy analysis by ELISA.

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Results

CC-292: A Potent, Highly Selective Btk Inhibitor

We have identified CC-292 (N-(3-(5-fluoro-2-(4-(2-methoxyethoxy)phenylamino)pyrimidin-4-ylamino)phenyl)acrylamide; Figure 1, Supplemental Figure 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 ($IC_{50\text{apparent}} < 0.5\text{nM}$, $K_{\text{inact}}/K_I = 7.69 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) and is highly selective (Supplemental Tables 1 and 2). 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 Cys 481 in Btk (EGFR, Itk, Jak3, Supplemental Table 3, Supplemental Table 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 Figures 2, 3 and 4 and Supplementary 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 ($EC_{50}=8 \text{ nM}$, Figure 2A and Supplemental Figure 5), phosphorylation of the Btk substrate, PLC γ 2, as well as activation of the downstream kinase, Erk, all previously shown to be sensitive to Btk inhibition (Di Paolo, et al., 2011; Honigberg, et al., 2010). Importantly, 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 (Figure 2A).

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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 one hour exposure to CC-292 continued to be suppressed ~8 hours in drug-free media (Figure 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 Figure 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 (Figures 3A, Supplemental Figure 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 Figure 8) and has demonstrated selectivity against the structurally related kinase EGFR ($IC_{50app} > 25$ nM), and upstream Src-family 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

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by CC-292 (Figure 3B). Results from this analysis can be reported in absolute values, such as the pg free Btk/ ug 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 to untreated samples and the extent of Btk bonded was demonstrated to be proportional to CC-292 drug concentration (Supplemental Figure 9). Importantly, 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 Figures 5, 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 also demonstrated in freshly isolated human primary naïve 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 (Figure 4A). Importantly, kinase inhibition and occupancy also reflected efficacy in B cell functional assays such as B cell proliferation (EC_{50} 3 nM; Figure 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 re-synthesis 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

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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 pre-dose Btk protein levels at 24 hours and 71% of pre-dose levels 48 hours after drug administration (Figure 5). Importantly, pharmacokinetic (PK) analysis of mouse plasma from this experiment indicated circulating CC-292 was absent in 5 of 6 animals by the 8 hour time point (data not shown). Presently, the potential contribution of active metabolites of CC-292 cannot be excluded. These data provide precise 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 (CIA) model of arthritis

The CIA model has been shown previously to respond to both B cell modulating therapies as well as direct Btk inhibition (Di Paolo, et al., 2011; Chang, et al., 2011; Honigberg, et al., 2010; Pine, et al., 2007; 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 3mg/kg respectively. (Figure 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 Figure 10). Importantly, CC-292 also demonstrated significant effects on the generation of inflammatory chemokines and cytokines in this model including KC, IL-6, and TNF 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, spleens collected either 2 or 24 hours after the last CC-292 dose were assayed for Btk occupancy. Occupancy in spleen lysates tracked closely with inhibition of the clinical signs of disease: 34%

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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) CC-292. Consistent with Btk re-synthesis 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 (Figure 6B). Morphologic and histopathologic analysis of 6 affected joints (4 paws, 2 knees) demonstrated a dose-dependent protection of 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 (Figure 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 vivo*. In addition, there was a strong correlation between the concentration of CC-292 required for Btk occupancy, inhibition of BCR signaling, and consequent functions such as B cell proliferation. As part of a larger clinical study with CC-292 in healthy adult human volunteers, we paired traditional pharmacokinetic analysis of plasma drug levels with Btk occupancy analysis in a B cell enriched fraction from freshly isolated human blood to determine the PK-PD relationship of CC-292 following single oral administration in humans. After initial dose escalation, 2 mg/kg CC-292 was found to be optimal for analysis of this PK-PD relationship. 6 healthy adult subjects were administered a single oral dose of CC-292 (2.0 mg/kg) and sequential blood samples were isolated over time to determine the

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relationship between the plasma concentration of CC-292 and Btk occupancy in an enriched B cell population.

2 mg/kg CC-292 was rapidly absorbed in all subjects with peak plasma concentrations achieved within 30-120 minutes after dose administration and a mean measured maximum plasma concentration of 542 ng/mL (C_{max}) was attained. Plasma concentrations declined to near or below the lower limit of detection (0.1 ng/mL) within 24 hours post dose with a median terminal elimination half-life of 1.9 hours. Plasma concentrations of CC-292 at 48 hours post dose were below the lower limit of quantification in all subjects (Figure 7).

Analysis of Btk occupancy was determined at each time point using the covalent probe ELISA. The absolute value of free Btk in lysates of enriched B cells isolated before CC-292 administration averaged 465 ± 67 pg free Btk/ug total protein (mean \pm SEM) for the 6 subjects administered CC-292 at a dose of 2.0 mg/kg. Within 4 hours after dose administration, 5 of 6 subjects had greater than 98% Btk occupancy with the 6th subject achieving 84% occupancy (Supplemental Table 7). Complete or near-complete Btk occupancy was sustained in all 6 subjects for between 8-24 hours post administration of CC-292 and this occurred at a time when plasma concentrations of CC-292 were low or approaching the limit of quantification. Thus, as in the experiment in mice described above, detection of free Btk over the ensuing 24-96 hour period post administration of CC-292 was a reflection of the re-synthesis rate of Btk by existing B cells plus the addition of any new B cells circulating in the periphery. Free Btk protein levels recovered towards 75% pre-dose values within 96 hours with a re-synthesis half-life of 48-72 hours and an average re-synthesis rate of 3.0 pg Btk/ug protein/hour. These data demonstrate an uncoupling of PD from PK and, similar to data generated in preclinical models, reveal that CC-292 action on Btk in human clinical trials was sustained for several hours after circulating drug levels declined to undetectable levels (Figure 7). The generation of metabolites was not evaluated in this clinical study and, therefore, the potential contribution of active metabolites of CC-292 cannot be excluded. These translational studies

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demonstrate the capability to precisely determine the concentration of CC-292 required for complete inhibition of Btk in human subjects to inform subsequent drug development.

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Discussion

Recent analysis suggests that attrition rates in clinical development are highest in Phase 2 clinical trials, where lack of PD activity and/or efficacy leads to failure rates of approximately a third of drug candidates (Kola and Landis, 2004). To address this, the development of novel strategies enabling earlier insights into PD activity is an intense area of translational research.

As a critical component of BCR signaling, Btk has emerged as an important drug target for the treatment of B cell disorders. CC-292 is a novel, potent, selective covalent inhibitor of Btk that has advanced rapidly into human clinical studies using an innovative translational medicine strategy to assess PD activity at each step of drug discovery into early clinical development.

Covalent inhibition of Btk has allowed creation of an assay to measure on-target activity, facilitating both preclinical research and clinical development by enabling a quantitative understanding of the relationship among dose, exposure, target engagement, functional consequence, and efficacy. The translational method described here quantitatively measures CC-292-Btk engagement by direct assay with an ELISA that sensitively detects the presence of drug-free Btk protein. In vitro, the concentrations of CC-292 required for inhibition of Btk activity and Btk occupancy were virtually equivalent ($EC_{50s} = 8$ vs. 6 nM, respectively) demonstrating a near stoichiometric relationship. In freshly isolated primary human B lymphocytes, there was a close correlation between the concentration of CC-292 required to inhibit Btk signaling, B cell proliferation, and achieve Btk occupancy, suggesting a quantitative relationship among inhibition of Btk kinase activity, target occupancy, and functional assays in vitro. This direct correlation supports the use of Btk occupancy as a surrogate marker for inhibition of Btk activity. Importantly, this relationship between the inhibition achieved and the extent of CC-292-target engagement measured by the covalent probe was maintained in vivo.

CC-292 demonstrated therapeutic efficacy in a mouse CIA model, with 85% and 95% inhibition of disease observed at doses of 10 mg/kg/day and 30 mg/kg/day, respectively. The reduced disease severity

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seen with CC-292 treatment recapitulates *xid* mice, which harbor an inactivating mutation in the Btk gene and have a reduced incidence and severity of CIA disease induction (Mangla, et al., 2004) as well as previous studies using pharmacological inhibition of Btk to reduce disease activity in autoimmune models (Honigberg, et al., 2010; Chang, et al., 2011; Di Paolo, et al., 2011; Liu, et al., 2011a). In addition to a full phenotypic response, once daily, oral dosing of 10 mg/kg CC-292 resulted in 84% Btk inhibition verified by Btk occupancy analysis assayed 2 hours after dose administration. Btk occupancy measured 24 hours after a 10 mg/kg dose suggested drug-free Btk protein was re-synthesized to approximately 60% of pre-dose levels. That sustained protection from the clinical signs of arthritis were provided at this dose suggests 100% Btk inhibition may not be required throughout a 24 hour time period and that intermittent inhibition of Btk may be sufficient for modulation of autoimmune disease in a clinical setting (Liu, et al., 2011a). To guide this determination, future studies with the translational Btk occupancy assay will enable establishment of the optimal dose, dose frequency, and degree of Btk inhibition required for full disease modification in animal models. Given the inhibitory activity on other members of the Tec family of kinases we cannot rule out the possibility of their inhibition playing a role in the efficacy seen *in vivo*.

As expected, CC-292 PK was dissociated from its PD *in vivo*, a feature confirmed by analysis of Btk occupancy. CC-292 remained active on Btk for a prolonged duration *in vivo* after plasma drug levels had declined to undetectable levels. Recovery from CC-292 treatment occurred slowly in both mice and humans as new Btk protein was made. In mice, where Btk occupancy was measured in spleen homogenates, Btk protein was re-synthesized to 50% of pre-dose levels 24-48 hours after a single dose. This differs from the re-synthesis of Btk protein seen in humans which required 48-72 hours to recover to 50% of baseline protein levels. This may reflect a more rapid re-synthesis rate of Btk in mice. However, this may also reflect the fact that in mice, Btk return was measured in spleen lysates whereas Btk re-synthesis in humans was measured in isolated peripheral B cells. We cannot rule out the possibility that either different cellular populations or B cell subsets resident in the spleen may synthesize Btk protein more quickly resulting in an increased re-synthesis rate in mice. In both mouse and human studies

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however, CC-292-Btk engagement persisted well after circulating drug had disappeared. In this way, covalent inhibitors allow a departure from the confines of traditional drug design; a pharmacokinetic profile that includes a long circulating half-life to ensure 24 hour target coverage is not necessary. Instead, a covalent inhibitor must achieve concentrations sufficient to engage all available molecular target only for a short period and then the re-synthesis rate of the target itself dictates the duration of drug action. As demonstrated here, the level and length of drug action can be empirically determined by target occupancy measurements to rationally adjust dosing.

The action of CC-292 on Btk was confirmed and PD was assessed in this first-in-human trial. This represents a marked acceleration of clinical PD evaluation, typically not available until Phase 2 clinical testing. The rapid identification of doses providing Btk target engagement provides an advantage in the design of subsequent human clinical trials and supports Phase 2 dose selection to incorporate safety, tolerability, and on-target activity. Future clinical trials in patients will appropriately assess the relationship of complete or partial Btk inhibition to therapeutic outcomes. However, direct quantification of Btk engagement in these trials will reduce uncertainty about the dose required for target inhibition and enable selection of the optimal pharmacological dose and dosing schedule. By providing this information to clinicians early in clinical development, sub-therapeutic drug administration in initial patient cohorts may be avoided and provide time efficiencies in clinical testing to more rapidly impact patient health. CC-292 has advanced to Phase 1b clinical testing in relapsed, refractory B cell malignancies with initial patient cohorts dosed at levels identified in this study. By refining the number of cohorts required for dose finding, it is anticipated that this trial will quickly provide recommended Phase 2 doses allowing rapid advancement into Phase 2 testing. Furthermore, CC-292 is the only Btk inhibitor to our knowledge with suitable safety profile to support its advance into clinical trials in patients with autoimmune disease and human clinical development of this molecule is occurring in parallel for both oncology and autoimmune indications.

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Given a long protein half-life, highly restricted expression pattern, and the presence of a poorly conserved cysteine in the ATP binding pocket, Btk represents an excellent target for selective covalent inhibition. The long protein half-life of Btk, shown previously to be > 12 hours in human primary B cells (Saffran, et al., 1994), provides for prolonged duration of drug action that extends well beyond the time frame of systemic covalent drug exposure. The uncommon cysteine targeted by CC-292 confers the opportunity for selective inhibition of Btk, as only 10 of the approximately 500 human kinases share placement of the cysteine in a homologous location within the ATP binding pocket. Importantly however, even in kinases sharing this cysteine, selectivity can be achieved with thoughtful drug design (Supplemental Table 3). Finally, since Btk is readily accessible in peripheral blood cells, target engagement can be measured easily by covalent probe ELISA. Although the translational approach described here is well suited to drug targets expressed and accessible in the hematologic system, there is the potential for broader utility. Certainly, direct measurement of drug-target engagement by covalent inhibition would be invaluable to assist all preclinical in vitro and in vivo work as long as the relevant tissue is accessible. For example, beyond the experiments described in this publication, the occupancy assay has been used to differentiate on-target vs. off-target effects in IND-enabling toxicology studies. Moreover, the PK-PD modeling attainable in preclinical models with this technique would promote informed dose selection as a program moves forward into a clinical setting. In this research, we have offered a powerful example demonstrating the potential and translational capabilities of covalent inhibition and believe this alternate approach to drug discovery has provided new opportunity for rapid and rational dose selections that will ultimately maximize patient benefit in clinical testing.

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Author contributions:

Designed and synthesized compounds CC-292 and CNX-500: RT, HM, JS, ZZ, RCP

Designed and performed experiments: SA, AD, MS, MN, EKE, MTL

Designed and oversaw in vivo animal models: RK, EKE, PC, WFW

Designed, oversaw and reviewed CC-292 phase 1a HNV clinical trial: SRW, HL, PC, EKE, WFW, MIF

Wrote or contributed to the writing of the manuscript: EKE, JS, WFW

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Figure Legends

Figure 1. Chemical Structure of CC-292 (N-(3-(5-fluoro-2-(4-(2-methoxyethoxy)phenylamino)pyrimidin-4-ylamino) phenyl)acrylamide

Figure 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 μ g/ml of the BCR ligand α -IgM. Btk autophosphorylation as well as Btk substrate phosphorylation (P-Y1217-PLC γ 2) and downstream activation of Erk were assayed by immunoblot. Quantitation of immunoblot demonstrated that CC-292 inhibits Btk autophosphorylation with EC₅₀ = 8 nM (n=4) (Supplemental Figure 4). B. Ramos cells were treated with compound for 1 hour. Cells were then resuspended in compound free media and stimulated with 5 μ g/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.

Figure 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 μ M 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 % Btk occupancy.

Figure 4. Btk Occupancy with CC-292 in Human Primary B cells Correlates with Inhibition of Btk Signaling and Inhibition of B Cell Proliferation. A. Human naïve B cells were isolated by negative selection, treated with the indicated concentrations of CC-292 for 1 hour, stimulated with 5 μ g/ml α -IgM

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for 10 minutes and lysed. Lysates were split in two for immunoblot and occupancy analysis. Representative immunoblot from 3 independent experiments shown and occupancy from 3 experiments plotted as mean \pm SD. B. Human naïve B cells (CD19+, IgD+) were purified from whole blood by negative selection with MACS purification. Purified naïve B cells were stimulated with 5 μ g/ml α -IgM for 72 hours. 3 H-Thymidine was added to the media for the final 16h and 3 H incorporation measured. Experiments were done in triplicate. The mean \pm standard deviation were plotted and the EC₅₀ calculated from n=3 separate experiments. CC-292 inhibits B cell proliferation EC₅₀ = 3 nM.

Figure 5. Btk Occupancy and Btk Protein Resynthesis Can Be Detected in Mice In Vivo. Mice were treated orally once with 50 mg/kg CC-292 to inhibit all Btk protein. At 4, 8, 16, 24, 48, 72, and 96 hours after treatment, spleens and serum were harvested (n=6 mice/timepoint). Spleens were homogenized and assayed on the covalent probe ELISA for Btk target site occupancy and compound concentration in serum was measured. Btk protein recovered to 50% pre-dose levels 24-48 hours after CC-292 administration. Pharmacokinetic analysis of compound concentration in plasma at each timepoint demonstrated compound was undetectable in 5/6 mice by the 8 hour timepoint. Plotting the mean plasma level of CC-292 in mice vs. the mean % Btk occupancy (both \pm SEM) demonstrates CC-292 action on Btk protein persists for >12 hours after circulating compound has disappeared.

Figure 6. CC-292 is Efficacious in an Established Collagen Induced Arthritis Model. A. Male DBA/1 mice were injected with bovine type II collagen in Freund's complete adjuvant on day 0 and day 21. On study days 25–27, onset of arthritis occurred, and mice were randomized into treatment groups (10/treatment group, 4/group for normal). Randomization into each group was done after swelling was obviously established in at least one paw. Treatment was initiated after enrollment. Treatment continued daily (QD at 24 h intervals) through arthritis day 14. CC-292 demonstrated dose-dependent inhibition of disease symptoms as measured by the daily clinical arthritis score plotted over 14 days of treatment. CC-292 administered at either 10 or 30 mg/kg was similar to dexamethasone control in inhibiting disease symptoms. **p<0.05 for 10 and 30 mg/kg CC-292 (ANOVA). In-life study conducted at Bolder

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Biopath, Boulder, CO, plotted as mean \pm SEM. B. Spleens were collected 2 and 24 hours following the final administration of CC-292 (n=6 mice per group). Lysates were made and run on the covalent probe ELISA to determine Btk occupancy. CC-292 at 3 mg/kg occupied 34% of the Btk at 2 hours; 10 and 30 mg/kg occupied 84-95% Btk at 2 hours, respectively. Occupancy shown for each individual mouse with mean of each group indicated by bar. C. Histopathologic analysis of the six joints in affected CIA mice demonstrated decreased cartilage and bone damage as well as inflammation and pannus in CC-292-treated animals. CC-292's inhibition of histopathologic signs of disease was significant ($p < 0.05$) and dose-dependent such that 10 and 30 mg/kg had effects similar to that of the positive control dexamethasone (Inhibition 82% with dexamethasone, 87% with 10 mg/kg CC-292, 96% with 30 mg/kg CC-292).

Figure 7. Pharmacokinetics and Pharmacodynamics are Uncoupled with Covalent Inhibitor CC-292 in Healthy Human Volunteers. PK analysis of 6 subjects dosed with CC-292 demonstrated rapid absorption and mean peak plasma levels (C_{max}) of 542 ng/mL CC-292. PD analysis of Btk target occupancy in the same 6 subjects displayed maximal occupancy at 4 hours (average cohort occupancy $>97\%$) with sustained occupancy through 24 hours and recovery of Btk protein levels towards 50% 48-72 hours after CC-292 administration. Mean \pm SEM for plasma level and % Btk occupancy depicted.

Figure 1.

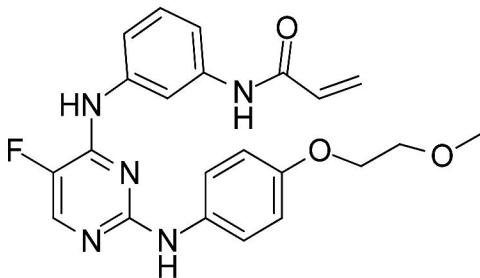


Figure 2.

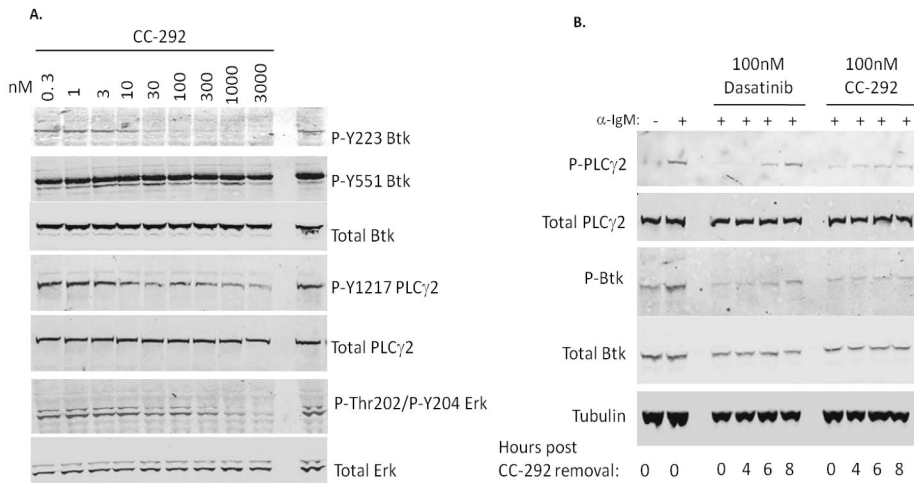
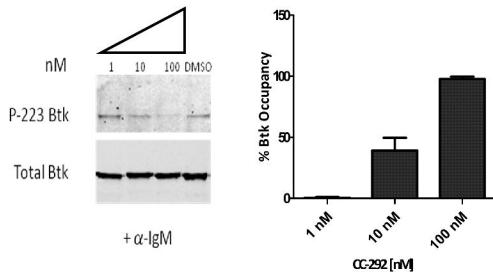


Figure 4.

A



B

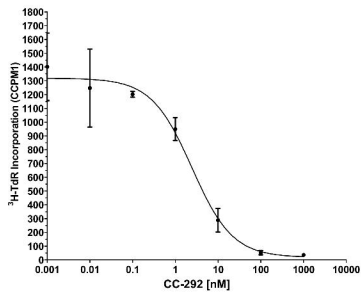


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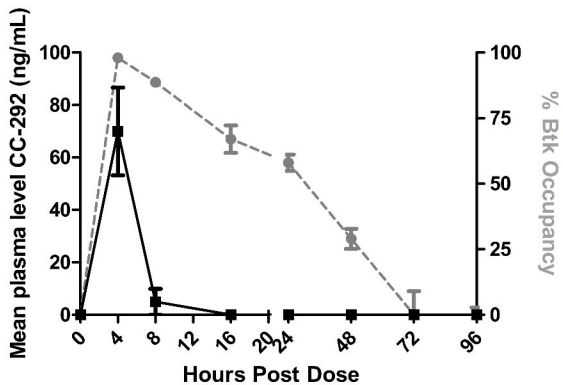


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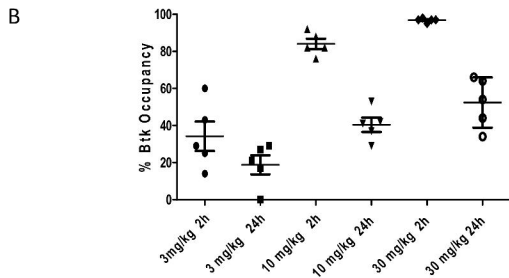
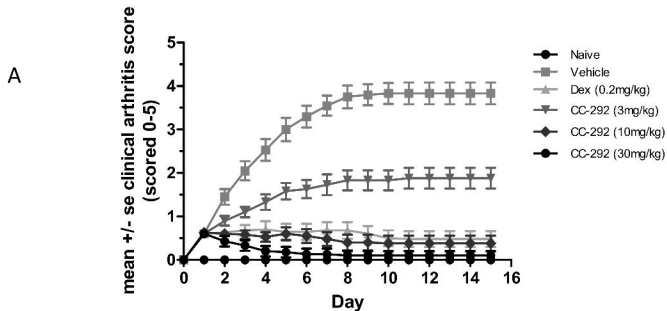


Figure 6.

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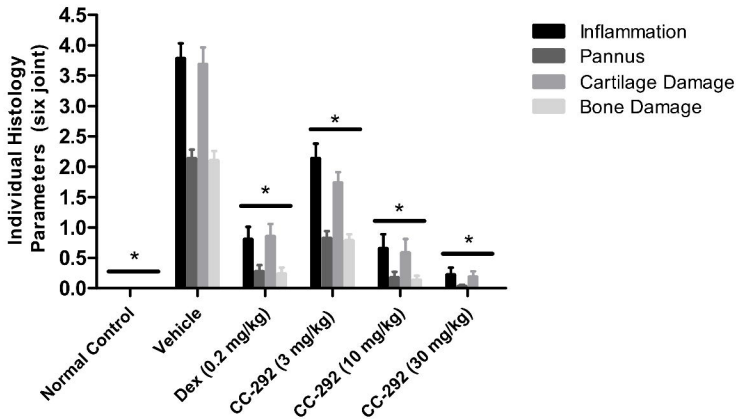


Figure 7.

