In Vitro Reactivity Assessment of Covalent Drugs Targeting Bruton's Tyrosine Kinase

#P211
October 17th, 2011

Matthew Labenski, Prasoon Chaturvedi, Erica Evans, Hormoz Mazdiyasni, Michael Sheets, Sharon Aslanian, Deqiang Niu, Mariana Nacht, Russell Petter, Juswinder Singh, William Westlin

Avila Therapeutics, Inc. 45 Wiggins Avenue, Bedford, MA 01730 USA

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Overview

Abstract:

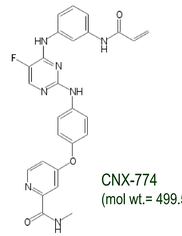
Covalent inhibitors have proven to be very successful therapies for a wide range of indications. To ensure that the potential for non-specific reactivity is minimized, all new covalent inhibitors should be screened against both the intended target as well as potential off-target proteins. Bruton's's tyrosine kinase (Btk) is a member of the Tec family of kinases that is involved in B-lymphocyte development, differentiation, and B cell receptor signaling. CNX-774 is a potent, selective, and orally available small molecule inhibitor of Btk that forms a ligand-directed covalent bond with Cys-481, a non-conserved amino acid within the ATP binding site of the enzyme. In this study, we have assessed the off-target reactivity and specificity of CNX-774, employing a panel of HPLC and LC-MS based assays. Biochemically, CNX-774 demonstrated potent inhibition of Btk with an IC50 of <1nM in an Omnia continuous-read assay. The covalent bonding of CNX-774 to Btk was confirmed by incubating recombinant Btk protein with a 10-fold molar excess of CNX-774 for 1 hour at room temperature and analysis by MALDI-TOF MS. A shift in protein mass corresponding to the molecular weight of CNX-774 confirmed the covalent bonding of CNX-774 to Btk. Digestion of the covalently bonded Btk with pepsin followed by MS/MS analysis established the bonding of CNX-774 to Cys-481. Cellular potency as well as prolonged duration of action of CNX-774 was demonstrated in Ramos cells by using a biotinylated covalent probe that targets the same Cysteine residue as CNX-774. In order to assess the reactivity and potential off-target non-specific binding of CNX-774, it was tested in a panel of assays that included glutathione (GSH) reactivity, plasma protein bonding in an albumin-depleted human plasma preparation, and general extractability in whole blood. Conjugation of CNX-774 with GSH did not occur after incubation with 5 mM GSH at pH 7.4 for 4 hours at 37ºc. Off-target protein reactivity was assessed by incubating CNX-774 with albumin-depleted human plasma at a final concentration of 0.1 mM for 1 hr at 37ºC, followed by analysis by MALDI-TOF MS. No change was observed in the molecular mass of any protein in the compound-treated plasma protein sample demonstrating that CNX-774 does not bond covalently to any of the high- to mid-level abundance human plasma proteins. The potential covalent bonding of CNX-774 to off-target proteins in blood was determined by incubating CNX-774 in fresh rat or human whole blood at 37ºC and monitoring the compound concentration over time by mass spectrometry. CNX-774 was found to be >90% extractable after 1 hr of incubation in both rat and human whole blood. These results demonstrate that CNX-774 has potent inhibitory activity towards the intended target, Btk, while achieving remarkable specificity in a variety of assays designed to assess off-target reactivity towards abundant cellular thiols and blood proteins. These studies demonstrate the utility of intelligently designed *in vitro* assays to determine specificity and off-target reactivity of Targeted Covalent Inhibitors at the discovery stage of the drug development process minimizing the potential for unintended non-specific reactivity.

Objectives:

To assess the *in vitro* reactivity of covalent inhibitors of *Btk* to minimize the potential for unintended non-specific reactivities, using CNX-774 as the representative compound.

Results:

- CNX-774 strongly inhibited Btk activity in Ramos cells with an IC_{50} of 1-10 nM
- CNX-774 demonstrated strong time- and dose-dependent occupancy of Btk in Ramos cells
- Complete modification of recombinant Btk was observed after a 1hr incubation at room temperature with 10-fold excess of CNX-774
- MS/MS confirmation of Cys 481 as the target amino acid for CNX-774 covalent modification
- Almost complete recovery of CNX-774 was observed after a 3 hr incubation with 5 mM GSH at physiological pH, indicating a low inherent reactivity of the compound.
- More than 90% of CNX-774 was recovered in both fresh rat and human blood.
- Upon incubation of CNX-774 with albumin-depleted human plasma, no change was observed in the molecular mass of any of the observed proteins, demonstrating high specificity.



CNX-774
(mol wt.= 499.5)

Methods:

Biochemical and Cellular activity:

Biochemical activity of CNX-774 was determined by OMNIA assay using recombinant full length Btk protein. Cellular activity against Btk was assessed in Ramos cells. The cells were treated with compound and then stimulated with the BCR ligand α-IgM. Btk autophosphorylation as well as Btk substrate phosphorylation (P-PLCγ2) were assayed by Western blot.

Evaluation of Covalent bonding to Btk:

Covalent Bonding:

Intact Btk (Invitrogen, 0.45 mg/mL) was incubated for 60 min at a 10-fold molar excess of compound to protein at room temperature. After the incubation, 5 µL aliquots of the samples were diluted with 15 µL of 0.2% TFA, and passed through a micro C4 ZipTip directly onto the MALDI target plate using sinapinic acid as the desorption matrix. Covalent Bonding was determined by monitoring the shift in the molecular weights of the compound treated and the untreated protein. Samples were analyzed on an AB Sciex 4800 TOF/TOF fitted with a CovalX HM2 detector.

Protein Digestion and Identification of CNX-774 Modified Amino Acid:

Recombinant Btk was incubated with CNX-774 as described above, following which the protein was precipitated with acetonitrile and washed 3 times with acetonitrile to remove excess compound. The protein was then subjected to a standard in-solution chymotrypsin digest, by first reduction with dithiothreitol, followed by alkylation with iodoacetamide, and digestion with chymotrypsin at 28ºC for 4hrs. The digest was finally quenched by addition of 2% TFA.

Five µL volumes of peptides were first trapped on a Proxeon trap cartridge (100 uM x 2cm C18). Peptides were then eluted and sprayed from a custom packed emitter (75uM x 25 cm C18) with a linear gradient from 100% solvent A (0.1% formic acid) to 35% solvent B (0.1% formic acid in Acetonitrile) in 35 minutes at a flow rate of 300 nanoliters per minute on a Proxeon Easy nanoLC system directly coupled to a Thermo LTQ Orbitrap Velos mass spectrometer. Data dependent acquisitions were set up according to an experiment where full MS scans from 350 Da-2000 Da were acquired in the Orbitrap FT at a resolution of 60,000 followed by 10 MS/MS scans acquired in the LTQ ion trap instrument. The raw data file was processed with Mascot Distiller (Matrixsciences, Ltd.) into peak lists and then searched against the human taxonomy of the SwissProt database using the Mascot Search engine (Matrixsciences, Ltd.). Parent mass tolerances were set to 5 ppm and fragment mass tolerances were set to 0.5 Da. The variable modifications of Acetyl (Protein N-term), pyro Glu for N-term Gln, and oxidation of Met, and Carbamidomethyl Cys were used along with the appropriate CNX compound adduct mass.

Evaluation of CNX-774 Off-Target Reactivity

Glutathione (GSH) Reactivity:

GSH reactivity was assessed by incubating 5 µM of CNX-774 with GSH (5 mM) at 37ºC with a pH of 7.4. At various designated time points, 5µL of the reaction mixture was injected onto a C8 column (Agilent Technologies, Zorbax 3.5µm SB-C18 2.1x30 mm) and analyzed with an AB Sciex QTrap 4000 mass spectrometer. Parent recovery was assessed and calculated as percent of the time zero value. Values shown in figure represent average of 3 injections.

Whole blood recovery:

CNX-774 was spiked into either fresh human or rat whole blood at a final concentration of 10 µM and placed in a shaking 37ºC incubator. At 1 hour 50 µL of whole blood was removed and proteins were precipitated with acetonitrile containing 1 µg/mL carbutamide as an internal standard. Samples were centrifuged at 16,000 x g for 10 minutes and supernatants were transferred to a plate for mass spec analysis. Concentration of CNX-774 was assessed by LC-MS/MS using an API 4000 Q Trap mass spectrometer.

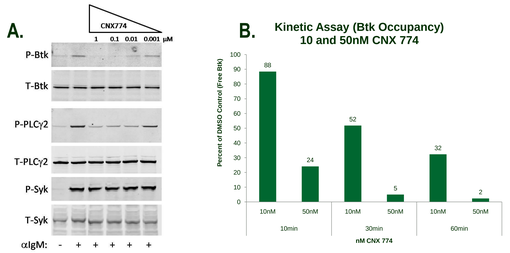
Plasma Protein Bonding:

Pooled human plasma was depleted of albumin using the SwellGel Blue Albumin Removal Kit (Pierce, Rockford, IL). The plasma was then diluted to a final concentration of 1mg/mL. 1 µL of a 1 mM compound solution was added to 10 µL of 1 mg/mL plasma proteins and incubated at room temperature for 1 hr. At the end of the incubation, 5 µL of each sample was combined with 15 µL of 0.2% ACN and purified using a C4 ziptip (Millipore). Ovalbumin was used as a protein internal standard. Samples were mixed with Sinapinic acid and analyzed on an ABSciex 4800 MALDI TOF/TOF mass spectrometer fitted with a CovalX HM2 detector.

Results:

CNX-774 strongly inhibits Btk

Figure 1: (A) CNX-774 potently inhibited Btk in the Ramos cell-based assay as indicated by an EC50 of 1-10 nM. (B) Occupancy of Btk was shown in Ramos cells using a biotinylated probe which covalently modifies the same residue as CNX-774.



CNX-774 Binds Covalently and Selectively to Btk

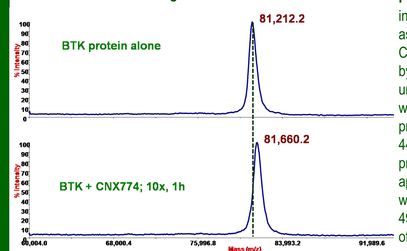


Figure 2: CNX-774 was incubated with Btk and analyzed as described under Methods. Covalent bonding was determined by comparing the mass of the untreated protein peak (top panel) with the mass of CNX-774 treated protein (bottom panel). A shift of 448 Da in the mass of the treated protein sample corresponding approximately to the molecular weight of CNX-774 (mol wt. = 499.5) indicated covalent binding of CNX-774 to Btk

CNX-774 Covalently modifies C481 of Btk

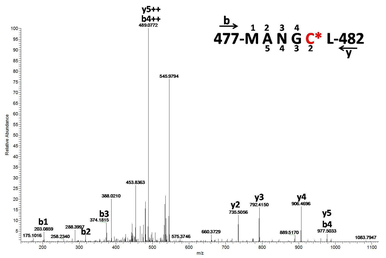


Figure 3: CNX-774 (10-fold molar excess) was incubated with recombinant Btk for 60 min at room temperature. Protein was then processed and digested with chymotrypsin as described in the Methods. Almost complete b and y ions coverage is observed confirming Cysteine 481 of Btk is the target amino acid covalently modified by CNX-774. No other modified CNX-774 peptides were identified in the digest.

CNX-774 Shows negligible reactivity towards GSH

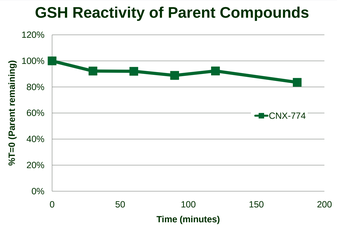


Figure 4: CNX-774 was incubated for 3 hrs in phosphate buffered saline in the presence of 5 mM GSH. Aliquots were drawn at various designated time points and analyzed for loss of CNX-774 as described in the methods section. More than 80% of the initial concentration of CNX-774 remains after a 3 hour incubation.

CNX-774 is stable in whole blood

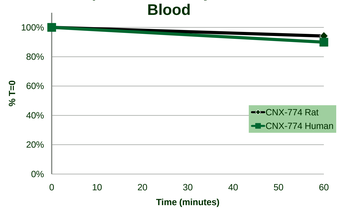


Figure 5: CNX-774 was incubated with either fresh human or rat whole blood as described in Methods. More than 90% of CNX-774 was recovered after 1 hour in either human or rat whole blood. These data demonstrate that CNX-774 is stable in whole blood and unreactive towards blood proteins, such as albumin and hemoglobin, among others.

CNX-774 does not bond to any of the mid-level abundance human plasma protein

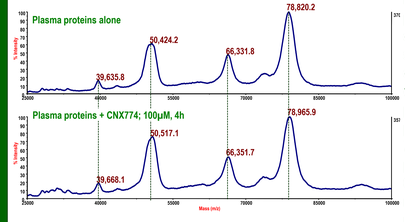


Figure 6: CNX-774 was incubated with human plasma proteins and analyzed as described under Methods. No significant change was observed in the molecular weights of any of the plasma proteins between the control protein (top panel) and the CNX-774 treated protein (bottom panel) samples indicating that CNX-774 did not covalently modify any mid-level abundance plasma protein.

Conclusions

- CNX-774 is a potent, irreversible and selective inhibitor of Bruton's Tyrosine Kinase
- CNX-774 specifically targets Cysteine 481 of Btk for covalent modification
- CNX-774 shows no measureable reactivity towards the most abundant intracellular thiol, Glutathione.
- CNX-774 is stable and non-reactive in fresh human and rat whole blood and does not covalently bond to any of the mid-level abundance human plasma proteins
- CNX-774 demonstrates potent inhibitory activity towards the intended target, Btk, while achieving remarkable specificity in a variety of assays designed to assess off-target reactivity towards abundant cellular thiols and blood proteins.