

## CITe-Id as a novel chemoproteomic platform to characterize covalent kinase inhibitors

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### Abstract

The therapeutic value of targeting protein kinases is shown by the little molecule inhibitors getting regulatory approval basically for disease treatment. Regardless of these victories, just a modest bunch of really specific inhibitors have been created for the almost 600 human kinases. The new endorsement of cysteine-coordinated covalent inhibitors of BTK and EGFR has reignited interest in covalent kinase therapeutics. One benefit of covalent medications is their capacity to intensely and for all time impair protein work regularly with just transient medication openness. We are centered around tests which covalently adjust individuals from the cyst-kinome, the subset of roughly 200 kinase which harbor a targetable cysteine buildup in nearness to the ATP restricting site. We have created quantitative mass spectrometry approaches which empower site-level cross examination of proteins focused by irreversible inhibitors on a proteome-wide scale. For singular tests which target kinases like EGFR, JNK, BMX, FGFR, CDK7, or BTK, we regularly recognize a few hundred intracellular protein targets.

We fostered a buddy, contest design examine called 'Refer to Id' which separates vague versus specific, focus subordinate inhibitor restricting to protein targets. Critically we effectively separate the collection of restricting focuses for tests which include primarily comparative analogs, proposing a productive instrument for restorative science advancement of second-age inhibitors. At long last, our quantitative methodology gives significant insights for advancement of inhibitors focusing on dark kinases. The mix of construction guided amalgamation educated by CITe-Id chemoformic target and site ID gives a versatile stage that conveys first-in-

class covalent substance tests that may fill in as helpful beginning stages for future little atom therapeutics.

### INTRODUCTION

Protein kinases oversee numerous parts of human physiology and are related as well as causatively connected to various human infections. Thus, they are alluring focuses for pharmacologic intercession, with most examination endeavors zeroed in on creating reversible, little atom kinase inhibitors. All the more as of late, irreversible covalent inhibitors have arisen as convincing other options. These mixtures for all time cripple kinase movement, ordinarily through covalent change of a nonsequence saved cysteine buildup that lies in or close to the ATP-restricting pocket. The clinical potential for covalent kinase inhibitors (CKIs) is exemplified by the new FDA endorsement of Ibrutinib, which targets BTK, and Afatinib, which targets EGFR.2 indeed, there are nearly 200 human kinases which range significant parts of the kinome phylogeny and harbor targetable, dynamic site-proximal cysteines ("cys-kinases"). We as of late depicted a progression of CKIs that specifically change cysteine deposits distal to the dynamic site ("far off cysteines"), with THZ15 and THZ531 as the most exceptional instances of this arrangement. These outcomes raise the charming chance that cysteine coordinated, specific CKIs might be created for a lot more extensive scope of the human kinome than recently imagined.

Notwithstanding these promising turns of events, it stays hard to anticipate cysteine reactivity, which addresses a bottleneck in the levelheaded plan of CKIs. All the more significantly, the potential for peculiar poison levels brought about by covalent alteration of off-target cysteines drives wariness for the wide utilization of irreversible inhibitors. Chemoproteomics, a subset of

mass spectrometry (MS) tests that consolidates the utilization of little atoms with the scientific force of proteomics, has been priceless for cross examination of CKIs and other test classes. For instance, ongoing chemoproteomic contemplates have tried to measure the reactivity of endogenous cysteines across the proteome;8 these information uncover a scope of profoundly responsive cysteine-thiols that address potential off-target liabilities for CKIs, and feature the need to incorporate objective site examinations as a component of covalent inhibitor improvement programs.

Couple Orthogonal Activity-based Protein Profiling (TOP-ABPP, and the quantitative isoTOP-ABPP) is a grounded approach that utilizes alkyne-derivatized tests to improve protein targets and recognize likely destinations of covalent adjustment. A significant impediment of this philosophy noted by the creators, was the trouble in getting site-level data when utilizing irreversible pharmacologic inhibitors, i.e., synthetically mind boggling and target particular mixtures. In this way, the current standard depends on little, nonselective cysteine tests as substitutes to profile the action of cysteine-coordinated specific pharmacologic inhibitors. This kind of backhanded, nonselective cysteine profiling doesn't officially affirm covalent ligand-target formation and may undersample low-plenitude/ - stoichiometry focuses because of the stochastic idea of LC-MS/MS information securing. Ongoing adjustments to the first methodology address a portion of these issues by utilizing fondness labeled CKIs to recognize off-targets and give a more complete image of potential poisonousness liabilities. Be that as it may, as detailed this procedure zeroed in on track distinguishing proof at the protein-level and thusly requires buddy biochemical measures to decide the specific site and covalent nature of ligand commitment.

We as of late exhibited that cysteine-coordinated tests and covalent medications share regular gas-stage separation way ways. Relevant to the limits noted over, the anticipated idea of these section particles can be utilized to improve peptide succession task including the particular site of covalent alteration. Here, we expand on these outcomes to set up another chemoproteomic stage that use fondness labeled analogs of pharmacologic CKIs for the biochemical improvement of focuses, alongside tunable peptide fractionation and custom phantom preparing to recognize inhibitor target locales. Our new

stage for Covalent Inhibitor Target-site Identification (CITe-Id) empowers further inclusion of cysteines adjusted by pharmacologic CKIs, while affirming covalent bond development and giving portion reaction information to inhibitor restricting at every cysteine-thiol. As an amazing verification of-of-idea, we utilized CITe-Id to recognize different, sudden off-focuses of our cyclin-subordinate kinase (CDK) inhibitor THZ1. These new targets included Cys-840 on Protein Kinase N3 (PKN3), an understudied AGC-type kinase connected to metastasis in forceful prostate tumors. Buildup level information from CITe-Id worked with our improvement of JZ128 as a covalent inhibitor of PKN3. We utilized JZ128 as an instrument compound to distinguish novel potential PKN3 substrates. Our work epitomizes the utility of CITe-Id to uncover new pharmacologically addressable cysteines and speed up advancement of specific, covalent inhibitors.

## MATERIALS AND METHODS

### Antibodies and Reagents

Antibodies were acquired from the accompanying sources:  $\alpha$ -PKN3 (Novus Biologicals, Littleton, CO),  $\alpha$ -CDK7 (Cell Signaling Technologies, Danvers, MA),  $\alpha$ -CCNK (Bethyl Laboratories, Montgomery, TX), PRKCQ (Thermo Fisher Scientific),  $\alpha$ -FLAG (Sigma),  $\alpha$ -PKN1 (Invitrogen, Carlsbad, CA),  $\alpha$ -PKN2 (Cell Signaling),  $\alpha$ -LIMK1 (Cell Signaling),  $\alpha$ -PIP4K2A (Cell Signaling),  $\alpha$ -PIP4K2B (Cell Signaling),  $\alpha$ -PIP4K2C (Sigma),  $\alpha$ -PIKFYVE (Millipore), and  $\alpha$ -RIPK2 (Novus Biologicals). Hotspots for provisions and reagents are as per the following: Activated Thiol Sepharose 4B dabs (GE Healthcare, Piscataway, NJ); Sep-pak tC18 100 mg 96-well plate (tC18; Waters, Milford, MA); SOLA C18 plate (SOLA C18; Thermo Fisher Scientific, Waltham, MA); Ni:NTA attractive agarose dabs (Qiagen, Valencia, CA); Ammonium bicarbonate (AMBIC; Sigma, St. Louis, MO); Triethylammonium bicarbonate support (TEAB; Sigma), Ethanol (EtOH; Sigma); Acetonitrile (Macron, Avantor Performance Materials, Center Valley, PA); tris(2-carboxyethyl)phosphine (TCEP; Sigma); S-Methylmethanethiosulfonate

(MMTS; Sigma); DL-Dithiothreitol (Sigma); Iodoacetamide (IAA; Sigma); Trifluoroacetic Acid (TFA; Pierce, Rockford, IL); formic corrosive (FA; Sigma). High-Capacity Streptavidin Resin (streptavidin pitch; Pierce). Kinase substrate library (Anaspec). Streptavidin-formed layers (Promega). Engineered peptides were created by utilization of Fmoc science and decontaminated by turned around stage HPLC. THZ1, THZ1-R, and THZ531 were incorporated as depicted.

## Chemical Synthesis

All solvents and reagents were utilized as gotten. For check of compound designs,  $^1\text{H}$  NMR spectra were recorded with a Varian Inova 500 NMR spectrometer and referred to dimethyl sulfoxide. Compound movements are communicated in ppm. Mass spectra were estimated with Waters Micromass ZQ utilizing an ESI source coupled to a Waters 2525 HPLC framework working backward mode with a Waters Sunfire C18 5  $\mu\text{m}$ ,  $4.6 \times 50 \text{ mm}^2$  segment. Cleaning of mixtures was performed with either a Teledyne ISCO CombiFlash Rf framework or a Waters Micromass ZQ preparative framework. The immaculateness was investigated on a previously mentioned Waters LC—MS Symmetry (C18 column,  $4.6 \times 50 \text{ mm}^2$ , 5  $\mu\text{m}$ ) utilizing an inclination of 5–95% methanol in water containing 0.035% trifluoroacetic corrosive (TFA). Point by point engineered plans and portrayal information are accessible in Supplementary Chemical Synthesis Details and else-where.

## Cell Culture and Cloning

All cell culture was performed utilizing standard procedures. HeLa S3 and HEK 293T cells were refined in DMEM with 10% FBS, PC3 cells were refined in RPMI1640 with 10% FBS. Cells were gathered utilizing a scrubber. The cells were centrifuged at 300g and

washed twice with PBS before cell pellets were frozen at  $-80^\circ\text{C}$ . Full length PKN3 cDNA with a N-terminal FLAG-HA tag was cloned into the pUC57 vector (GenScript U.S.A., Piscataway, NJ). Simultaneously, a point transformation was presented at position 2886 (nearby grouping: ggccacacagggt) from a  $\rightarrow$  to create the C840S freak PKN3. Both PKN3 builds were extracted from pUC57 with EcoRI and XhoI and embedded into a pCDH-CMV spine.

## Target Engagement Pulldown Assays

HeLa S3 or PC3 cells were treated with expanding centralizations of either DMSO, THZ1, THZ1-R, JZ128, or JZ128-R for 4 h. Cells were collected by scratching and washed twice with PBS before lysis with NP-40 lysis support (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% v/v glycerol, 0.5% v/v NP-40, protease inhibitors). Protein content was estimated by BCA. Tests were treated with 2  $\mu\text{M}$  of either THZ1-DTB or JZ128-DTB for 18 h. at  $4^\circ\text{C}$ . Streptavidin sap was added and brooded for 1 h. at room temperature. The globules were washed multiple times with NP-40 lysis cradle. Bound protein was eluted by adding SDS-PAGE stacking support and bubbling for 5 min before Western smearing. 1% of test input was utilized for stacking controls.

## PKN3 Phospho Motif Identification

To decide the substrate theme of PKN3, in vitro kinase tests were performed with recombinant PKN3 on the peptide substrate library within the sight of ATP[ $\gamma$ - $^{32}\text{P}$ ]. These responses were completed in Kinase Assay Buffer I (SignalChem) at  $30^\circ\text{C}$  for 90 min. The peptides, which are biotinylated at their C-ends, were smeared onto streptavidin-formed layers and imaged with a Typhoon FLA 7000 phosphorimager. Itemized data on the convention is given somewhere else. The spot densities from the smudge cluster were evaluated and standardized by each line. These qualities were utilized to score the amino corrosive succession encompassing each recognized phosphosite

## RESULTS

Motivated by the growing clinical impact of CKIs, juxtaposed with key concerns related to potential toxicities as described above, we looked to foster CITE-Id as a strong chemoproteomic procedure that would go past protein-level ID of targets, and give a buildup level direct readout of focus subordinate covalent restricting of a given CKI. We recently portrayed explicit gas-stage discontinuity pathways for peptides covalently adjusted by cysteine-coordinated, irreversible inhibitors and clinical medications, including our as of late depicted CDK7/12/13 inhibitor, THZ1. Expanding on these bits of knowledge, we initially explained THZ1 with a desthiobiotin proclivity handle (THZ1-DTB) and afterward utilized Western blotch to affirm fixation subordinate marking and advancement of CDK7. Starting with a comparable contest design hatching, we planned CITE-Id to readout portion subordinate restricting of particular, pharmacologic inhibitors dependent on the investigation of altered peptides.

We performed streptavidin pulldown after tryptic processing of protein lysate to give an exceptionally improved pool of THZ1-DTB named peptides for ensuing encoding with iTRAQ reagents, trailed by multidimensional chromatography custom fitted for the hydrophobic idea of inhibitor-adjusted peptides and furthermore giving wide adaptability to fractionation profundity. Moreover, we expanded our past structure for ID of covalent inhibitor-changed peptides to represent the desthiobiotin fondness tag and linker, and affirmed improved grouping scores for ~85% of THZ1-DTB named destinations. At long last, we utilized peptide iTRAQ columnist particle forces to compute a serious portion reaction for inhibitor restricting at singular cysteine buildups.

## DISCUSSION

Although historically eschewed by drug development programs, irreversible inhibitors are now gaining momentum. We and others have utilized design guided blend to create particular CKIs which hinder kinase action through covalent change of nonconserved, dynamic site cysteine deposits. We as of late stretched out this worldview to accomplish specific hindrance by focusing on "distant" cysteine deposits. Reassuringly these outcomes propose that the scene of medication targets amiable to specific covalent restraint through cysteine reactivity might be altogether bigger than recently anticipated. Notwithstanding, our failure to foresee potential off-target reactivity addresses a significant risk for improvement of covalent medications. In this way, plan, improvement, and portrayal of covalent inhibitors should be considered with regards to the whole proteome.

Activity based protein profiling, exemplified by TOP-ABPP has given important understanding to the cell action of an assortment of little particles. Notwithstanding, in the particular instance of CKIs, these strategies ordinarily depend on the utilization of wanton cysteine tests as substitute readouts for the reactivity of specific inhibitors. Interestingly, our CITE-Id stage repurposes specific CKIs as proclivity reagents to straightforwardly advance protein targets. Besides, fuse of our new information on gas-stage fracture conduct normal for covalent test peptide adducts empowers us to zero in only on CKI-adjusted peptides. With CITE-Id we go around the troublesome errand of comprehensively profiling an enormous arrangement of cysteine deposits limited by an organically superfluous ABPP test to distinguish the humble arrangement of buildups which are limited by a specific inhibitor. Significantly, CITE-Id information give direct proof of covalent system of-activity while the fuse of multiplexed isotope names gives a readout of cutthroat portion reaction at each CKI-altered cysteine-thiol.

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