Biomedical Data Mining

Extended Abstract

A Chemoproteomic Strategy for Direct and Proteome-Wide Covalent Inhibitor Target-Site Identification

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Abstract

Despite recent clinical successes for irreversible drugs, potential toxicities mediated by unpredictable modification of off-target cysteines represents a serious hurdle for expansion of covalent drug programs. Understanding the proteome-wide binding profile of covalent inhibitors can significantly accelerate their development; however, current mass spectrometry strategies typically do not provide an immediate, amino acid level readout of covalent activity for complex, selective inhibitors. Here we report the development of CITe-Id, a completely unique chemoproteomic approach that employs covalent pharmacologic inhibitors as enrichment reagents together with an optimized proteomic platform to directly quantify dose-dependent binding at cysteine-thiols across the proteome. CITe-Id analysis of our irreversible CDK inhibitor THZ1 identified dose-dependent covalent modification of several unexpected kinases, including a previously unannotated cysteine (C840) on the understudied kinase PKN3. These data streamlined our development of JZ128 as replacement selective covalent inhibitor of PKN3. Using JZ128 as a search compound, we identified novel potential PKN3 substrates, thus offering an initial molecular view of PKN3 cellular activity. CITe-Id provides a strong complement to current chemoproteomic platforms to characterize the selectivity of covalent inhibitors, identify new, pharmacologically addressable cysteine-thiols, and inform structure-based drug design programs.

INTRODUCTION

Protein kinases govern many aspects of human physiology, and are associated and/or causatively linked to numerous human diseases. As a result, they are attractive targets for pharmacologic intervention, with most research efforts focused on developing reversible, small molecule kinase inhibitors. More recently, irreversible covalent inhibitors have emerged as compelling alternatives. These compounds permanently disable kinase activity, typically via covalent modification of a nonsequence conserved cysteine residue that lies in or near the ATP-binding pocket. The clinical potential for covalent kinase inhibitors (CKIs) is exemplified by the recent FDA approval of Ibrutinib, which targets BTK, and Afatinib, which targets EGFR. In fact, there are some 200 human kinases which span major branches of the kinome phylogeny and harbor targetable, active site-proximal cysteines “cys-kinases”. We recently described a series of CKIs that selectively modify cysteine residues distal to the active site (“remote cysteines”), with THZ15 and THZ5316 as the most advanced examples of this series. These results raise the intriguing possibility that cysteine-directed, selective CKIs may be developed for a much broader range of the human kinome than previously envisioned.

Despite these promising developments, it remains difficult to predict cysteine reactivity, which represents a bottleneck within the rational design of CKIs. More importantly, the potential for idiosyncratic toxicities caused by covalent modification of off-target cysteines drives skepticism for the broad use of irreversible inhibitors. Chemoproteomics, a subset of mass spectrometry (MS) experiments that mixes the utilization of small molecules with the analytical power of proteomics, has been invaluable for interrogation of CKIs and other probe classes. For instance, recent chemoproteomic studies have sought to quantify the reactivity of endogenous cysteines across the proteome; these data reveal a range of highly reactive cysteine-

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thiols that represent potential off-target liabilities for CKIs, and highlight the need to include target-site analyses as part of covalent inhibitor development programs.

Tandem Orthogonal Activity-based Protein Profiling (TOP-ABPP, and the quantitative isoTOP-ABPP) is a well-established approach that employs alkyne-derivatized probes to enrich protein targets and identify likely sites of covalent modification. An important limitation of this methodology noted by the authors, was the difficulty in obtaining site-level information when using irreversible pharmacologic inhibitors, i.e., chemically complex and target selective compounds. Thus, the current standard relies on small, nonselective cysteine probes as surrogates to profile the activity of cysteine-directed selective pharmacologic inhibitors. This type of indirect, nonselective cysteine profiling does not formally confirm covalent ligand-target conjugation and may undersample low-abundance/-stoichiometry targets due to the stochastic nature of LC-MS/MS data acquisition. Recent modifications to the original approach address some of these issues by using affinity-tagged CKIs to identify off-targets and provide a more complete picture of potential toxicity liabilities.18,19 However, as reported this strategy focused on target identification at the protein-level and therefore requires companion biochemical assays to determine the exact site and covalent nature of ligand engagement.

We recently demonstrated that cysteine-directed probes and covalent drugs share common gas-phase dissociation path-ways. Pertinent to the limitations noted above, the predictable nature of these fragment ions can be used to improve peptide sequence assignment including the specific site of covalent modification. Here, we build on these results to establish a new chemoproteomic platform that leverages affinity-tagged analogs of pharmacologic CKIs for the biochemical enrichment of targets, along with tunable peptide fractionation and custom spectral processing to identify inhibitor target sites. Our new platform for Covalent Inhibitor Target-site Identification (CITe-Id) enables deeper coverage of cysteines modified by pharmacologic CKIs, while confirming covalent bond formation and providing dose-response data for inhibitor binding at each cysteine-thiol. As a powerful proof-of-concept, we used CITe-Id to identify multiple, unexpected off-targets of our cyclin-dependent kinase (CDK) inhibitor THZ1. These new targets included Cys-840 on Protein Kinase N3 (PKN3), an understudied AGC-type kinase linked to metastasis in aggressive prostate tumors. Residue-level data from CITe-Id facilitated our development of JZ128 as a covalent inhibitor of PKN3. We used JZ128 as a tool compound to identify novel potential PKN3 substrates. Our work exemplifies the utility of CITe-Id to reveal new pharmacologically addressable cysteines and accelerate development of selective, covalent inhibitors.

RESULTS

Developing and Optimizing CITe-Id (Covalent Inhibitor Target-site Identification)

Motivated by the growing clinical impact of CKIs, juxtaposed with key concerns related to potential toxicities as described above, we sought to develop CITe-Id as a robust chemoproteomic strategy that would go beyond protein-level identification of targets, and provide a residue-level direct readout of concentration-dependent covalent binding of a given CKI. We previously described specific gas-phase fragmentation pathways for peptides covalently modified by cysteine-directed, irreversible inhibitors and clinical drugs, including our recently described CDK7/12/13 inhibitor, THZ15. Building on these insights, we first elaborated THZ1 with a desthiobiotin affinity handle (THZ1-DTB) and then used Western blot to confirm concentration-dependent labeling and enrichment of CDK7 (Supporting Information, SI). Beginning with similar competition-format incubation, we designed CITe-Id to readout dose-dependent binding of selective, pharmacologic inhibitors based specifically on the analysis of modified peptides. We performed streptavidin pulldown after tryptic digestion of protein lysate to provide a highly enriched pool of THZ1-DTB

labeled peptides for subsequent encoding with iTRAQ reagents, followed by multidimensional chromatography tailored for the hydrophobic nature of inhibitor-modified peptides and also providing wide flexibility for fractionation depth.23–25 In addition, we extended our previous framework for identification of covalent inhibitor-modified peptides to account for the desthiobiotin affinity tag and linker, and confirmed improved sequence scores for ~85% of THZ1-DTB labeled sites20 (Table S1). Finally, we used peptide iTRAQ reporter ion intensities to calculate a competitive dose-response for inhibitor binding at individual cysteine residues.

Our CITe-Id analysis of THZ1/THZ1-DTB identified 527 unique cysteine residues which were covalently modified by THZ1-DTB and detected reproducibly across biological replicates . Quantitative, competitive dose-response data from CITe-Id revealed that THZ1-DTB binding was independent of THZ1 concentration for a majority (>95%) of modified cysteine residues. These sites, which include highly abundant proteins such as glycolytic enzymes and tubulin, as well as many cysteines annotated as “highly reactive”,8 likely represent nonspecific probe binding. Against this null-distribution we identified dose-dependent competitive binding of THZ1 to eight cysteine residues, including the known targets CDKs 7, 12, and 13. In addition to these positive control data, we identified dose-dependent THZ1 competitive binding on several unexpected kinase targets, PKN3 at C840, protein kinase C theta (PRKCQ) at C661, and glycogen synthase kinase 3B (GSK3B) at C14. We next repeated CITe-Id, using THZ531, a THZ1 analog with reported selectivity for CDK12/13 over CDK7,6 as the competing, native inhibitor. These data recapitulated the known CDK selectivity profiles, and further demonstrate that THZ531 has little or no reactivity against the potential new targets of THZ1, PKN3, PRKCQ, or CYP1B1 while gaining some activity against GSK3B. These results confirm the ability of CITe-Id to quantitatively distinguish the target landscape of structurally similar inhibitors.

THZ1 Inhibits PKN3 via Covalent Binding to C840

One of the kinases targeted by THZ1 was PKN3, a kinase that has been functionally linked to metastasis and tumor growth, and is the target of a liposomal siRNA-based therapeutic currently in clinical trials (NCT0093857426).

Despite these data and clinical interest, PKN3 is understudied compared to other disease-associated kinases, which motivated us to further investigate it as a THZ1 target. A comparison of structural data for CDK7 and PKN3 (Swiss- Model Q6P5Z2) revealed that C840 of PKN3 is positioned close to the kinase active site, similar to the “remote cysteine” C312 of CDK7 targeted by THZ1. The targeted cysteine of CDK12 and PRKCQ are similarly positioned. We therefore sought to validate PKN3 as a covalent target of THZ1 and explore the potential of developing selective tool compounds to study PKN3 biology.

To confirm target engagement as well as the covalent nature of PKN3-THZ1 conjugate formation in vivo, we treated HeLa S3 cells with increasing concentrations of THZ1, using the reversible analog (THZ1-R5) as a negative control. After cell lysis we incubated protein extracts with THZ1-DTB, followed by streptavidin pulldown and detection of bound proteins by Western blot. As expected, THZ1 showed strong competition for CDK7, with similar reactivity profiles observed for CDK12/13 (probing for their obligate cyclin K binding partner as a proxy), as well as two new targets identified by CITe-Id, PKN3 and PRKCQ. The reversible analog THZ1-R competed poorly for binding to all targets.

We next sought to confirm that covalent modification of PKN3 by THZ1 inhibited enzyme activity. Using an in vitro kinase assay, we observed that THZ1 inhibited PKN3 in a fixed time-point format with an apparent IC50 of 72 nM, while the reversible analog was more than 50-fold less potent. We also confirmed that inhibition of PKN3 by THZ1 was time dependent, consistent with a covalent mechanism-of-action. A

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similar assay showed THZ1 to have weak potency against PRKCQ. To demonstrate that C840 is essential for THZ1 binding to PKN3, we expressed tandem FLAG and HA-tagged wild type (WT) or C840S (CS) mutant PKN3 in a clonal PC3 cell line with CRISPR-Cas9-mediated deletion of endogenous PKN3 (PC3 PKN3 KO). PC3 is a prostate cancer cell line that has been used previously to investigate PKN3 biology. Lysates were treated with THZ1-DTB, followed by streptavidin pulldown or immuno-precipitation with HA antibody. We found that the C840S mutation abrogated THZ1-DTB binding to PKN3. Collectively these data validate PKN3 as a new target of THZ1, and further demonstrate that pharmacologic activity is mediated by the covalent binding to a single cysteine (C840) on the kinase.

DISCUSSION

Although historically eschewed by drug development programs, irreversible inhibitors are now gaining momentum.41 We and others have used structure-guided synthesis to develop selective CKIs which inhibit kinase activity through covalent modification of nonconserved, active-site cysteine residues.42 We recently extended this paradigm to achieve selective inhibition by targeting “remote” cysteine residues.5,6 Encouragingly these results suggest that the landscape of drug targets amenable to selective covalent inhibition via cysteine reactivity may be significantly larger than previously predicted.3 However, our inability to predict potential off-target reactivity represents a major liability for development of covalent drugs. Therefore, design, development, and characterization of covalent inhibitors must be considered in the context of the entire proteome.8

Activity based protein profiling, exemplified by TOP-ABPP has provided valuable insight for the cellular activity of a variety of small molecules.8,11,13,14,16,17 However, in the specific case of CKIs, these techniques typically rely on the use of promiscuous cysteine probes as surrogate readouts for the reactivity of selective inhibitors.8,9,11,13–15,17 In contrast, our CITe-Id platform repurposes selective CKIs as affinity reagents to directly enrich protein targets. Moreover, incorporation of our new knowledge of gas-phase fragmentation behavior characteristic of covalent probe-peptide adducts20 enables us to focus exclusively on CKI-modified peptides. With CITe-Id we circumvent the difficult task of exhaustively profiling a large set of cysteine residues bound by a biologically irrelevant ABPP probe to identify the modest set of residues which are bound by a selective inhibitor. Importantly, CITe-Id data provide direct evidence of covalent mechanism-of-action while the incorporation of multiplexed isotope labels provides a readout of competitive dose–response at each CKI-modified cysteine-thiol.

It should be noted that binding data from CITe-Id is not directly analogous to inhibition of enzymatic activity, with CITe-Id supplementing but not replacing traditional enzyme-activity assays. Overall, CITe-Id is a complementary methodology that fits well within an irreversible inhibitor development program as it offers information valuable for understanding the precise mechanism of action as well as proteome-wide, covalent off-target effects.

Taken together, CITe-Id represents a conceptually novel strategy for rapid and accurate proteome-wide identification of covalently modified sites targeted by irreversible inhibitors. We expect that CITe-Id profiling will enable rapid irreversible inhibitor screening to inform medicinal chemistry optimization efforts as well as mechanistic studies of these inhibitors and their off-target effects. As illustrated by our PKN3 work, CITe-Id analysis can accelerate discovery of novel selective inhibitors and functional characterization, especially in the context of the understudied kinome.

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