LC-HRMS Based Label Free Screening Platform for Lysine-targeting Covalent Inhibitors

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Abstract

Covalent inhibitors, with irreversible target engagement, have become pivotal in drug discovery. Liquid chromatography-high-resolution mass spectrometry (LC-HRMS) is uniquely suited for high-throughput screening of these molecules, enabling label-free detection of native proteins and covalent adducts, precise quantification of target engagement through exact mass analysis, rapid characterization of binding kinetics (e.g., kinact/KI values) via intact protein analysis, and further binding site identification by peptide mapping analysis.

While cysteine-targeting covalent inhibitors are well-studied, their therapeutic scope is restricted by cysteine's low natural abundance and limited proximity to functional sites (e.g., kinase catalytic domains). Lysine, in contrast, offers greater abundance and broader targeting potential, addressing resistance mechanisms caused by cysteine modifications. However, lysine's ε-amino group (pKa~10.4) remains protonated at physiological pH, requiring microenvironmental pKa modulation or highly reactive electrophilic warheads for effective covalent binding. The principle of MS enables direct characterization of covalent binding through molecular weight shift detection, with sensitivity minimally affected by binding efficiency. Crucially, MS facilitates precise binding site identification: MS2 fragment ion analysis not only pinpoints covalently modified amino acid residues but also enables semi-quantitative comparison of multi-site binding efficiencies through relative site occupancy rates. This capability provides critical data for establishing site occupancy-efficacy correlations.

To address these challenges, we developed a label-free LC-HRMS platform for direct protein-level quantification of lysine covalent binding. Using NU6300 (a CDK2 inhibitor targeting Lys89) as a model, this method streamlines high-throughput screening of lysine-targeting inhibitors, bypassing labeling steps while maintaining analytical rigor.

In this study, the reaction system was maintained at a final CDK2 protein concentration of 1.0 μM, the serially diluted concentrations of positive control nu6300 ranged from 31.25 μM to 2000 μM, and the total incubation time was 60 min. The intensity of the native protein and covalent adducts were determined using UPLC-HRMS coupled with a C4 column.

A relatively high binding rate was observed by intact mass analysis. kinact/KI value were fitted using more than six concentrations and six timepoints. This orthogonal mass spectrometry strategy establishes a robust integrated screening platform that concurrently addresses three critical aspects of covalent drug development: covalent inhibitors screening, binding site identification and kinact/KI measurement.

Objectives

The Intact Protein Mass Spectrometry (Intact-MS) technique enables precise determination of a protein's molecular weight, with the capacity to detect specific mass shifts resulting from covalent modifications by various compounds. A novel Intact-MS screening strategy was established using CDK2 proteins and NU6300(a CDK2 inhibitor targeting Lys89). And a peptide mapping method was constructed to characterize the covalent binding site. Subsequently, we characterized the time-dependent target inactivation kinetics by determining the second-order rate constant (kinact/KI) through progress curve analysis. Crucially, this intrinsic parameter - unlike IC50 values affected by assay conditions - enables quantitative comparison of covalent inhibitor efficiency independent of concentration and incubation time, thereby providing critical insights for inhibitor optimization.

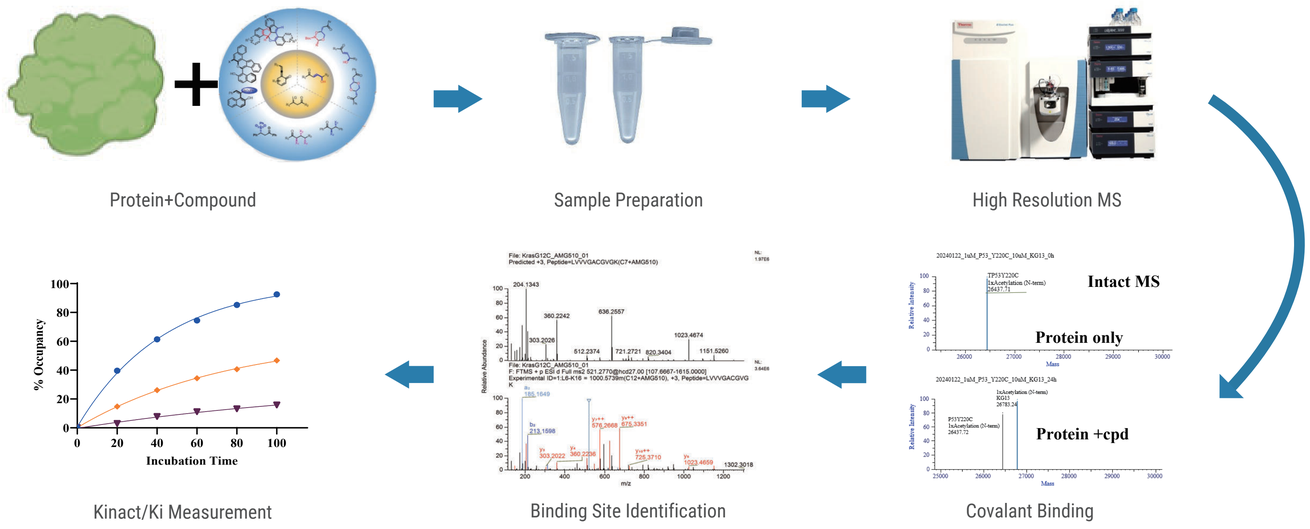

Figure 1. Covalent binding screening strategy

Methods and Results

Protein samples (1.0 μM) were incubated with NU6300 (31.25–2000 μM) under kinetic control conditions (60 min total duration, sampled at t = 5, 10, 15, 30, 45, and 60 min). Quantitative analysis of covalent adduct formation was performed using high-resolution mass spectrometry (Thermo Scientific Q Exactive Plus) equipped with a C4 reversed-phase column, with covalent complex abundance quantified via BioPharma Finder software. The occupancy rate at different incubation times were calculated using the following equation:

$$\%\ \text{Ocupancy} = \frac{I_{mol}}{I_{unmol} + I_{mol}} \times 100\%$$

Kinetic analysis revealed high target engagement (75.2% modification efficiency) when CDK2 (1.0 μM) was incubated with 2000 μM NU6300 for 60 min (Figure 2).

Apart from the intact mass, the amino acid sequence and the specific covalent binding site of the CDK2 was also characterized by peptide mapping using MS2 data. And the exact covalent binding site was located at K89, which was consistent with the published data (Figure 3).

The time-dependent inactivation kinetics were analyzed through pseudo-first-order approximation by correlating observed rate constants (kobs) with inhibitor concentrations. Temporal occupancy data were modeled using a single exponential decay function to derive kobs values at each concentration, followed by linear regression analysis of kobs versus [inhibitor] to determine kinetic parameters. This classical approach yielded a second-order rate constant kinact/KI = 242 $M^{-1}s^{-1}$ (Figure 4).

1. Covalent binding analysis

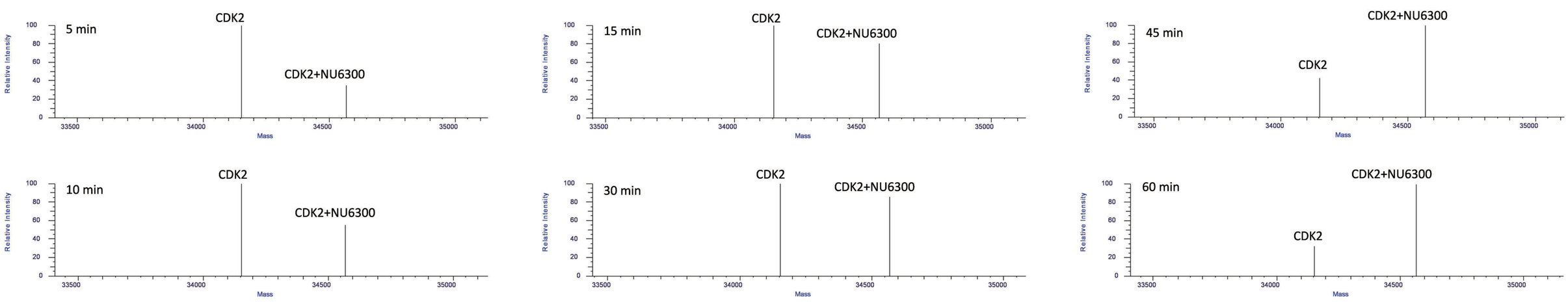

Figure 2. Covalent binding of a CDK2 by highly potent inhibitor NU6300, quantified by intact protein MS

2. Biding site identification

Peptide Coverage Map of Covalent Inhibitor Binding Site

Figure 3. Characterization of the binding site of NU6300 and CDK2

3. kinact/KI measurement

Figure 4. Determination of kinact/KI for irreversible covalent inhibitor NU6300 of CDK2

Conclusions

The resurgence of targeted covalent inhibitors in modern drug discovery has established the second-order rate constant (kinact/KI) as the preferred metric for quantifying covalent inhibition potency. We herein establish a high-resolution mass spectrometry-driven analytical platform for rapid kinetic characterization of irreversible inhibitors. Benchmarked with CDK2, this label-free methodology achieves determination of both occupancy rate and binding site of lysine-targeting covalent binding through intact protein and peptide level.

References

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