LC-HRMS BASED LABEL FREE SCREENING PLATFORM FOR COVALENT INHIBITORS

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Abstract

Covalent inhibitors have emerged as indispensable components in drug discovery and therapeutic development, with the irreversible nature of those target engagement positioning liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) as a powerful approach for high-throughput screening of these molecular entities.

LC-HRMS enables label-free direct detection of both native proteins and their covalent adducts within samples, while enabling relative quantification of target engagement through exact mass analysis. Intact protein analysis for rapid adduct confirmation facilitated covalent binding kinetics characterization, and determination of critical biochemical parameters such as K_{inact}/KI values.

In this study, the reaction system was maintained at a final $KRAS^{G12C}$ protein concentration of 1.5 μM, the serially diluted concentrations of positive control MRTX849 ranged from 0.64 μM to 4.00 μM, and the total incubation time was 100 seconds. The LC-HRMS platform demonstrated its multi-dimensional analytical capability by successfully quantifying EGFR (1 μM) target engagement with a covalent small-molecule drug conjugate (SMDC, 1 μM featuring a cysteine-reactive warhead). 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. K_{inact}/KI value were fitted using more than six concentrations and five timepoints. This orthogonal mass spectrometry strategy establishes a robust integrated screening platform that concurrently addresses two critical aspects of covalent drug development: covalent inhibitors (including SMDC) screening and K_{inact}/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 GDP-loaded KRASG12C and MRTX849, as well as EGFR and SMDC. Subsequently, we characterized the time-dependent target inactivation kinetics by determining the second-order rate constant (K_{inact}/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 scheme.

Methods and Results

Protein samples (1.5 μM) were incubated with MRTX849 (0.49–4.00 μM) under kinetic control conditions (100 s total duration, sampled at t = 0, 20, 40, 60, 80, and 100 s). 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 complete target engagement (100% modification efficiency) when KRASG12C (1.5 μM) was incubated with 4.0 μM MRTX849 for 100 sec (Figure 2). 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 K_{inact}/KI = 5.06×10^4 $M^{-1}s^{-1}$ (Figure 3).

Intriguingly, the EGFR-SMDC interaction exhibited biphasic covalent binding: EGFR-small molecule complex and EGFR-SMDC complex, with an occupancy% of 47% and 14% separately (Figure 4). These data enabled quantitative determination of the released warhead percentage through comparative analysis of the binding populations.

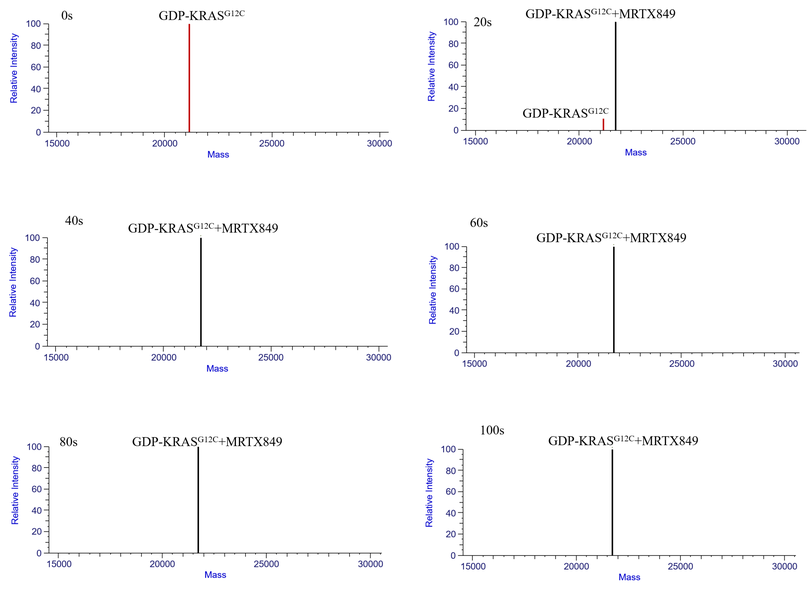


Figure 2. Covalent binding of GDP-$KRAS^{G12C}$ by highly potent inhibitor MRTX849, quantified by intact protein MS.

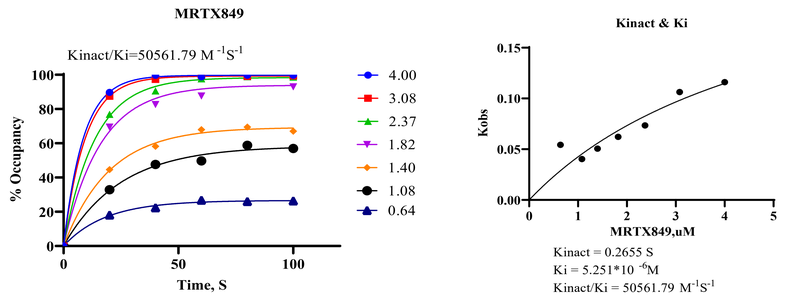
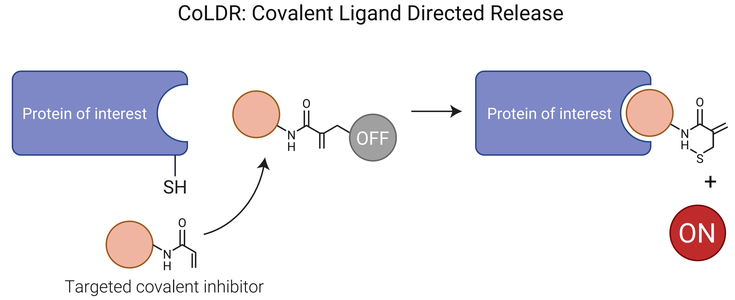


Figure 3. Determination of K_{inact}/KI for irreversible covalent inhibitor MRTX849 of GDP-$KRAS^{G12C}$.



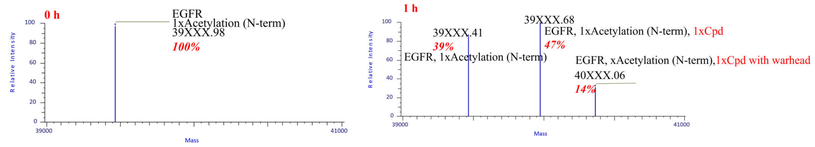


Figure 4. Covalent binding of EGFR by SMDC, quantified by intact protein MS.

Conclusions

The resurgence of targeted covalent inhibitors in modern drug discovery has established the second-order rate constant (K_{inact}/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 GDP-$KRAS^{G12C}$ and EGFR, this label-free methodology achieves determination of both conventional inhibitors adduct and SMDC adduct through intact protein analyses.

References

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