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Abstract

Protein kinases are central regulators of cellular signaling and have become prominent targets in drug discovery. Achieving selectivity among closely related kinases can pose a significant challenge due to their high structural similarity. While traditional reversible inhibitors often lack sufficient selectivity across highly homologous proteins, covalent inhibitors can offer improved target discrimination by reacting with non-conserved, nucleophilic residues like cysteines (Cys).

Here, we explore covalent hit finding for the vaccinia-related kinase 1 (VRK1) aimed at achieving selectivity over its closely related paralog VRK2. We established a mass spectrometry-based workflow that combines intact protein analysis with cellular cysteine mapping to characterize the kinetics and site specificity of covalent VRK1 inhibitors. This integrated approach enables quantitative assessment through determination of k_{inact}/K_i values and allows precise identification of cysteine modification sites in a cellular context. A one-step covalent inhibition mechanism with $K_i > 100$ mM is insufficient to characterize this class of inhibitors.

Using this method, we identified Cys50 – one of six cysteines in VRK1 – as the primary site of covalent modification for identified inhibitor series. Mutation of Cys50 and Cys68 to alanine or serine led to a marked reduction in inhibitor activity, indicating functional relevance. Further, peptide mapping confirmed selective modification of P-loop residing Cys50 in VRK1 derived from cells. The corresponding residue in VRK2 (Leu42) is not reactive therefore providing isoform selectivity. Altogether, these findings demonstrate the value of MS-based profiling for identifying and guiding the discovery of covalent and paralog selective kinase inhibitors.

4. Cys50 is as Major Covalent Labeling Site.

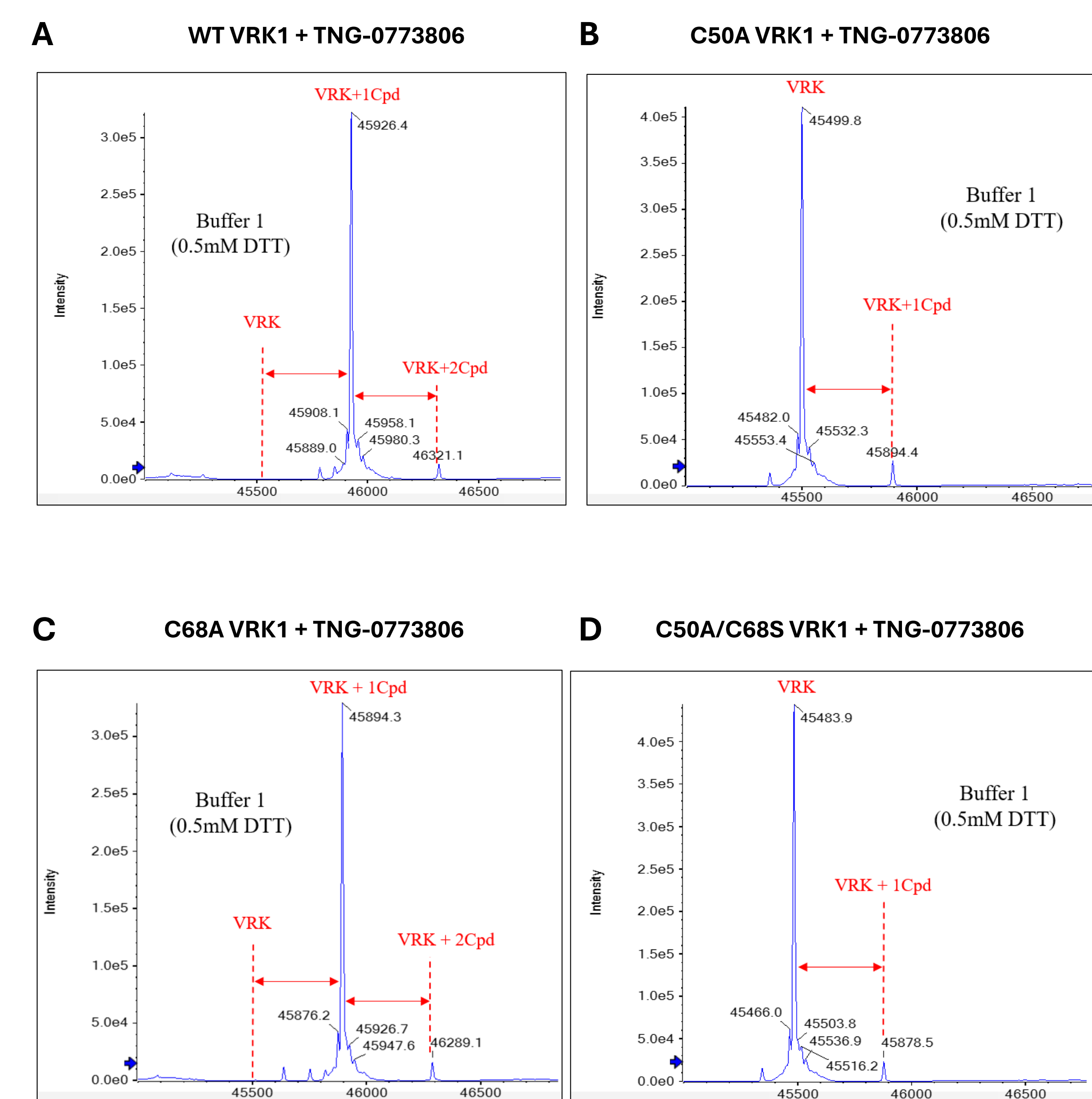


Figure 4. Covalent adduct formation of TNG-0773806 with VRK1 and its cysteine mutants. (A) Incubation of wild-type (WT) VRK1 with TNG-0773806 yields a predominant single-peak adduct, indicating specific covalent interaction. (B) The C50A single mutant abolishes adduct formation, suggesting Cys50 is the primary covalent binding site. (C) The C68A single mutant retains adduct formation, indicating Cys68 is not involved in covalent binding with TNG-0773806. (D) The C50A/C68S double mutant also fails to form an adduct.

1. Workflow for Identification and Characterization of Covalent VRK1 Inhibitors

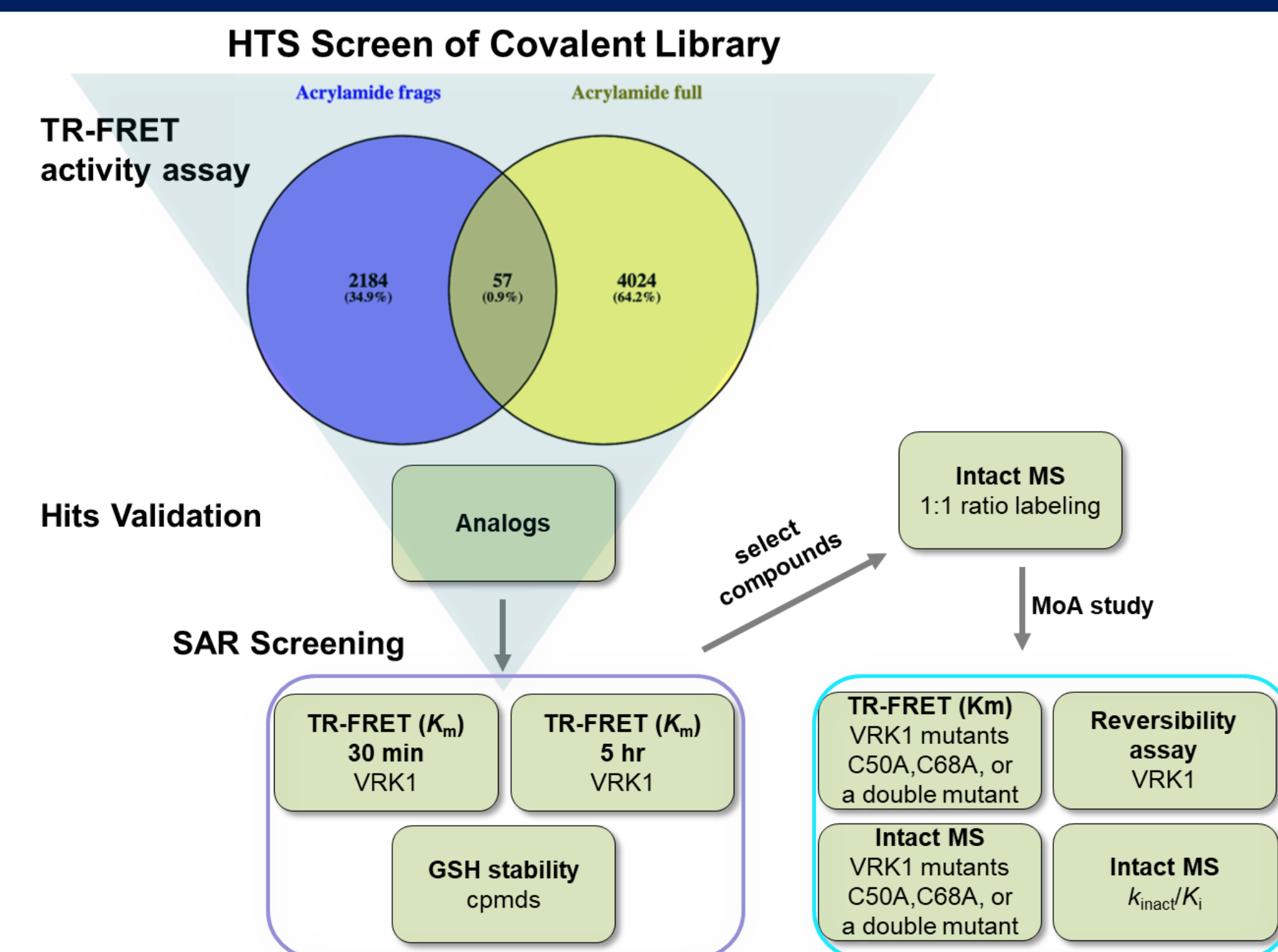


Figure 1. The schematic illustrates the workflow for screening and profiling acrylamide-based covalent inhibitors targeting VRK1. Initial TR-FRET screening of both acrylamide fragments (frags) and full-acrylamide compounds yielded overlapping hits.

5. In-Cell MS Confirms Cys50 Reactivity on VRK1

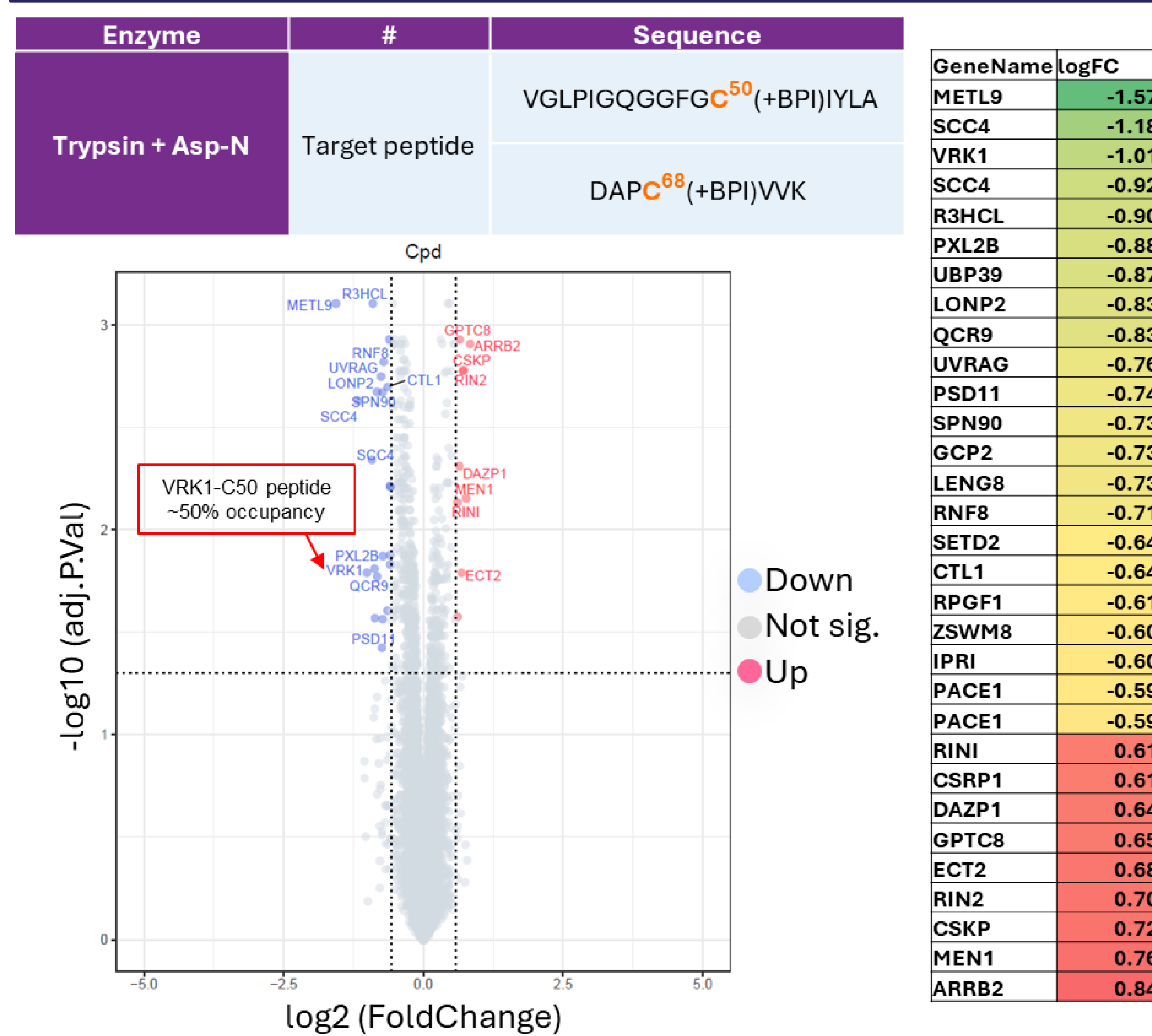


Figure 5. Chemoproteomic prioritization and evaluation of covalent-allosteric targeting of VRK1. VRK1 is ranked among the top differentially enriched proteins based on chemoproteomic profiling, highlighting its ligandability. TNG-0784293 and U251MG cell line were used for the assay.

2. Covalent Inhibitor Engages at an Allosteric Site in VRK1 Structure

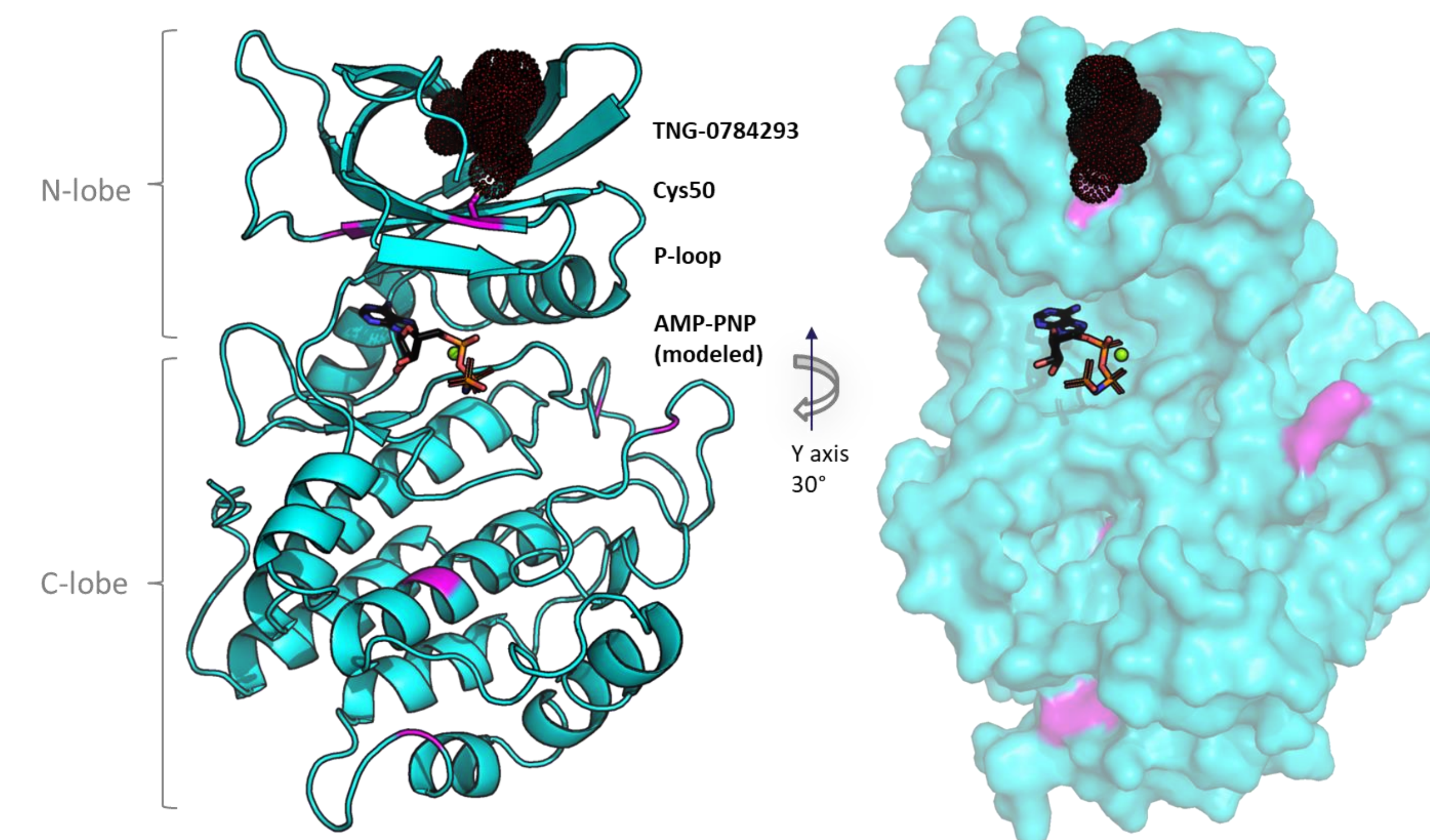


Figure 2. Structural analysis of VRK1 and its covalent inhibitor binding site. (A) The covalent inhibitor TNG-0784293 is shown as dots; cysteine residues for binding are colored in magenta; Cys50 and the modeled ATP analog AMP-PNP are displayed as sticks. (B) Surface representation showing the covalent inhibitor binds to an allosteric pocket distinct from the ATP-binding site.

3. Covalent Inhibitors of VRK1 Exhibit Time-Dependent Inhibition Profiles

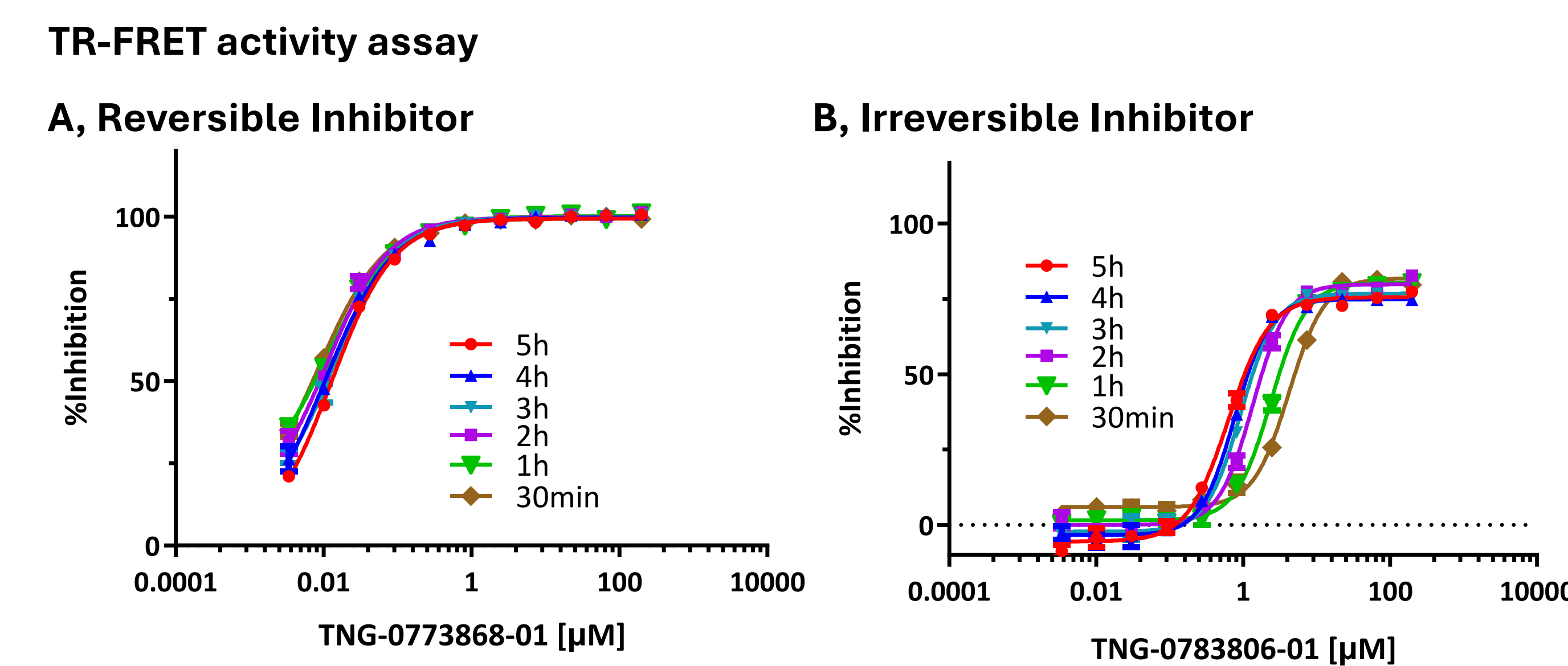


Figure 3. Time-dependent inhibition profiles of reversible and covalent VRK1 inhibitors. (A) Incubation of a reversible VRK1 inhibitor shows minimal change in IC_{50} values, indicating time-independent inhibition. (B) In contrast, a covalent cysteine-targeting inhibitor exhibits a time-dependent leftward shift in IC_{50} , consistent with irreversible binding kinetics.

6. Intact MS Enables Kinetic Profiling of Covalent Inhibitors

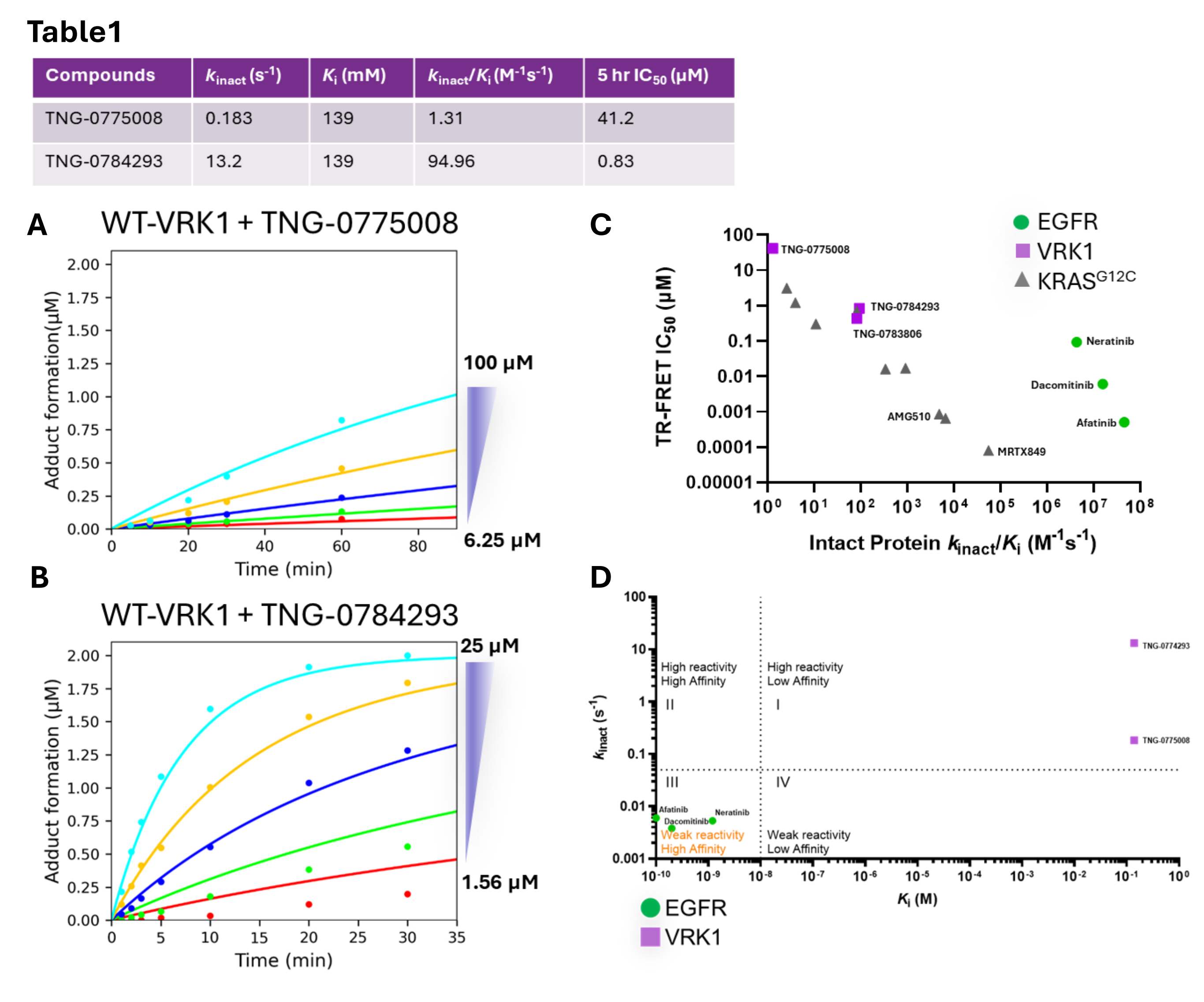
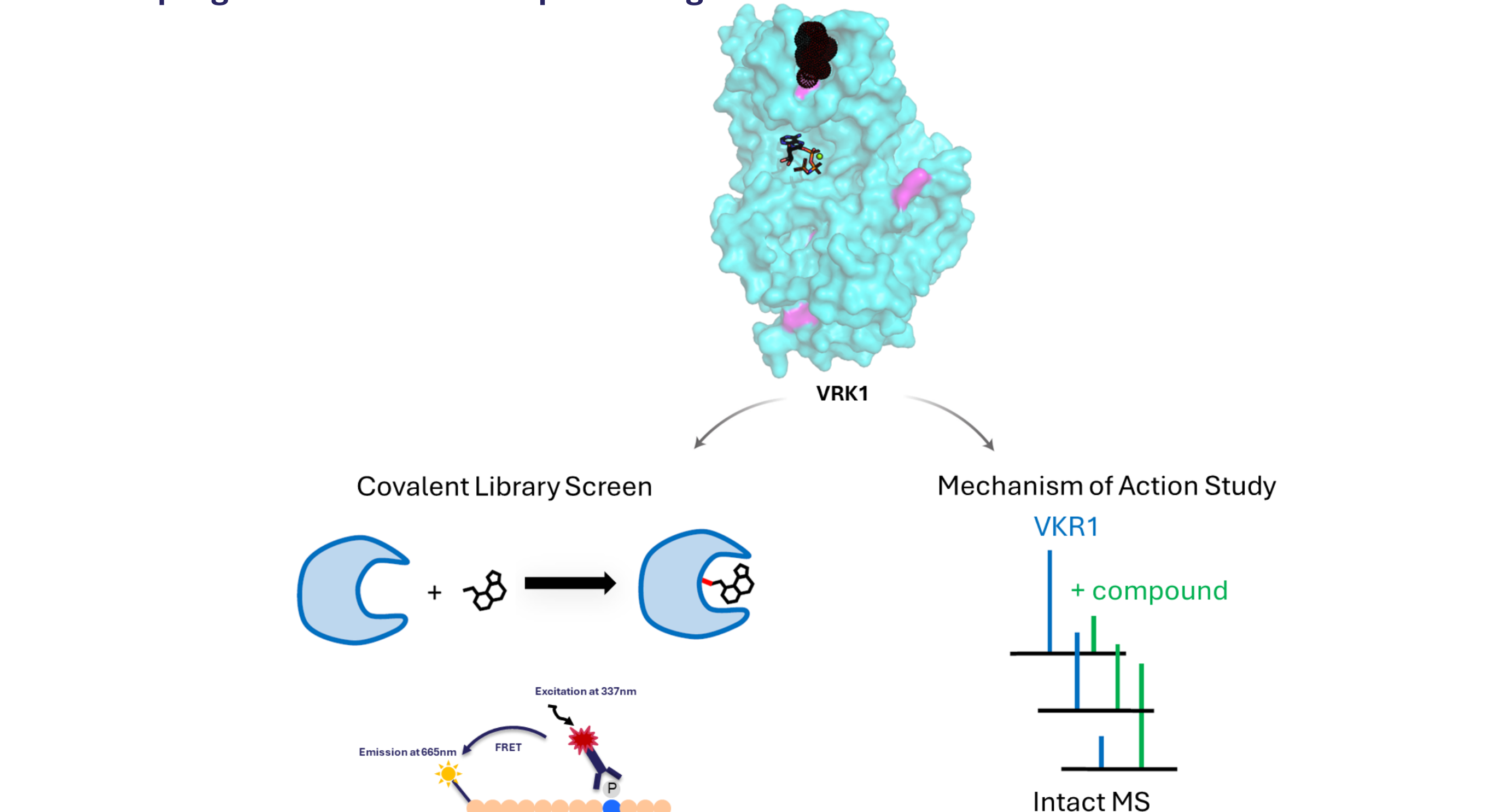


Figure 6. Kinetic characterization of covalent VRK1 inhibitors and correlation with TR-FRET potency. (A) and (B) k_{inact} and K_i was determined TNG-0775008 and TNG-0784293 (C) Correlation analysis between TR-FRET IC_{50} values and intact protein k_{inact}/K_i ($M^{-1}s^{-1}$) reveals a strong inverse relationship for VRK1 compared to reference EGFR kinase inhibitors. (D) VRK1 covalent hits displayed very weak apparent K_i , indicating limiting binding affinity for optimization. Table 1. Summary of kinetic parameters and biochemical potency of TNG-0775008 and TNG-0784293 against VRK1.

Summary

- Using a focused covalent library, intact protein MS, and cellular cysteine mapping, we identified compounds that selectively engaged Cys50 among VRK1 cysteines.
- Mutagenesis studies (C50A, C68A, and double mutant) showed loss of adduct formation only when Cys50 was removed, validating its primary labeling site.
- Cys50 provides a potential selectivity handle over the VRK2 paralog, which lacks a reactive cysteine at the analogous site.
- Our study demonstrates that mass spectrometry-based profiling can effectively identify covalent, isoform-selective inhibitors of VRK1.
- Collectively, these results support the potential discovery of covalent, paralog-selective VRK1 inhibitors targeting this allosteric cysteine (Cys50).
- VRK1 program is available for partnering.



Acknowledgements

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References

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