Mass Spectrometry-Based Kinetic and Site-Specific Profiling of Covalent Inhibitors Targeting VRK1

Mu-Sen Liu, Ph.D.

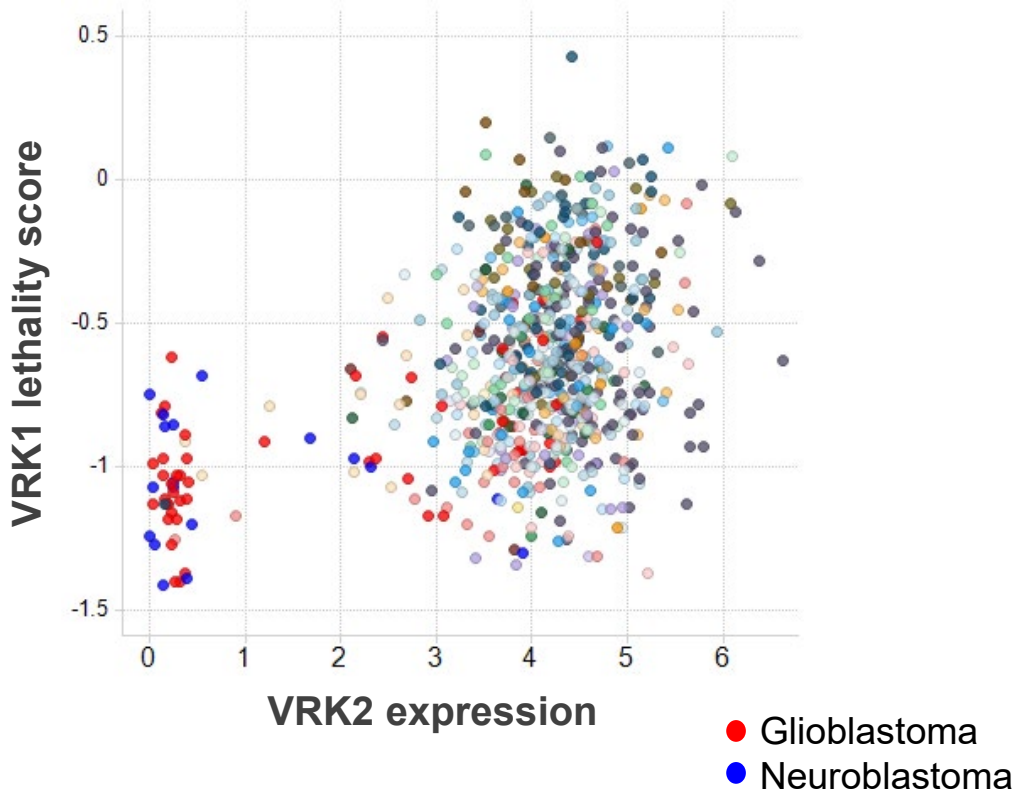
2026.10.22

4th Annual
Covalent Drug Discovery & Development Summit
Advancing Novel Covalent Therapies to the Clinic

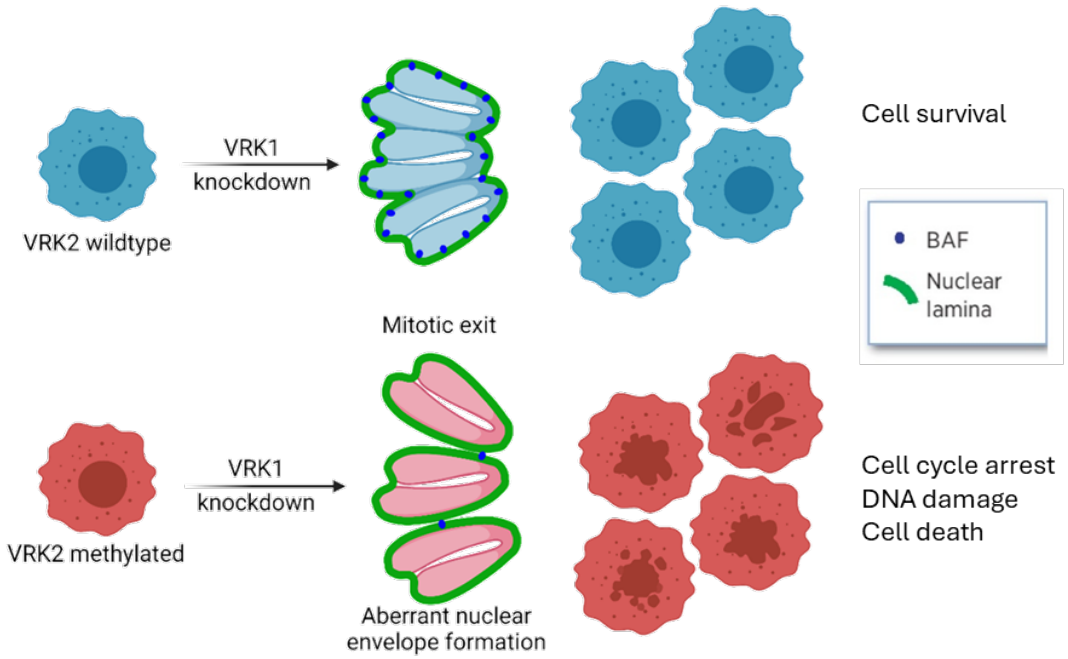
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VRK1 is a synthetic lethal kinase target in VRK2-low brain cancer

Approximately 66% of malignant brain tumors have low VRK2 expression



VRK1 knockdown leads to cell cycle arrest, accumulation of DNA damage, and cell death



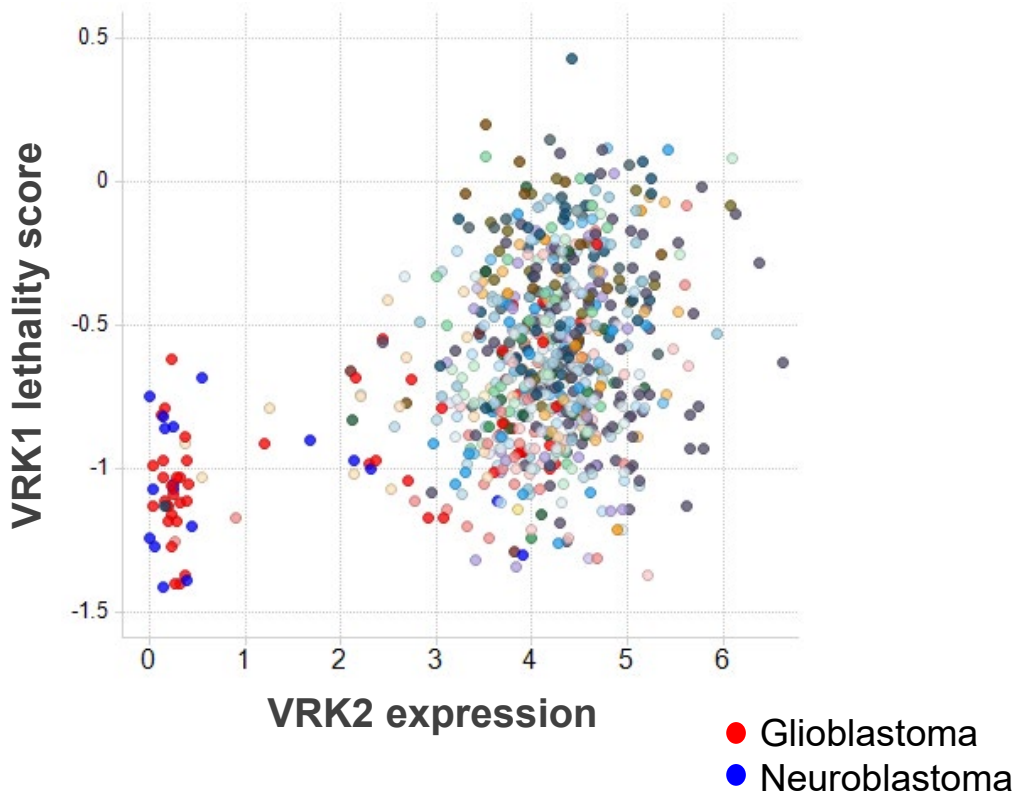
Shields, J. A. *et al. Cancer Res.* **82**, 4044- 4057 (2022).

In **VRK2-low cancers** (such as promoter-methylated glioblastomas and neuroblastomas), cell survival becomes entirely dependent on the sister kinase **VRK1**.

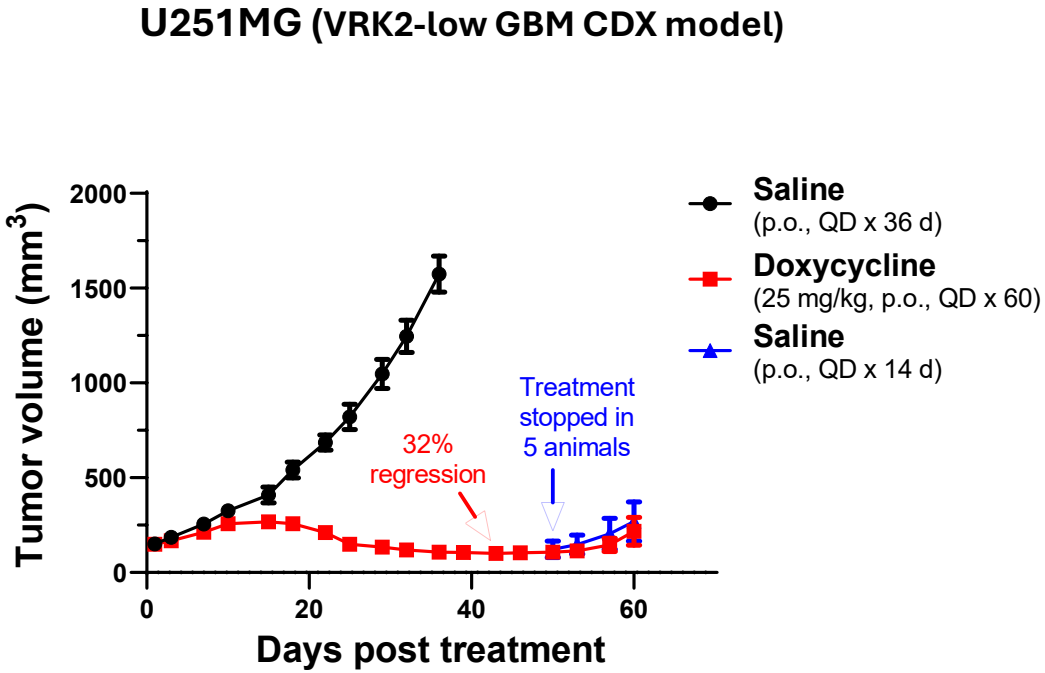
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VRK1 is a synthetic lethal kinase target in VRK2-low brain cancer

Approximately 66% of malignant brain tumors have low VRK2 expression



Dox-inducible CRISPRi-based VRK1 knockdown drives tumor regressions in VRK2-low tumors



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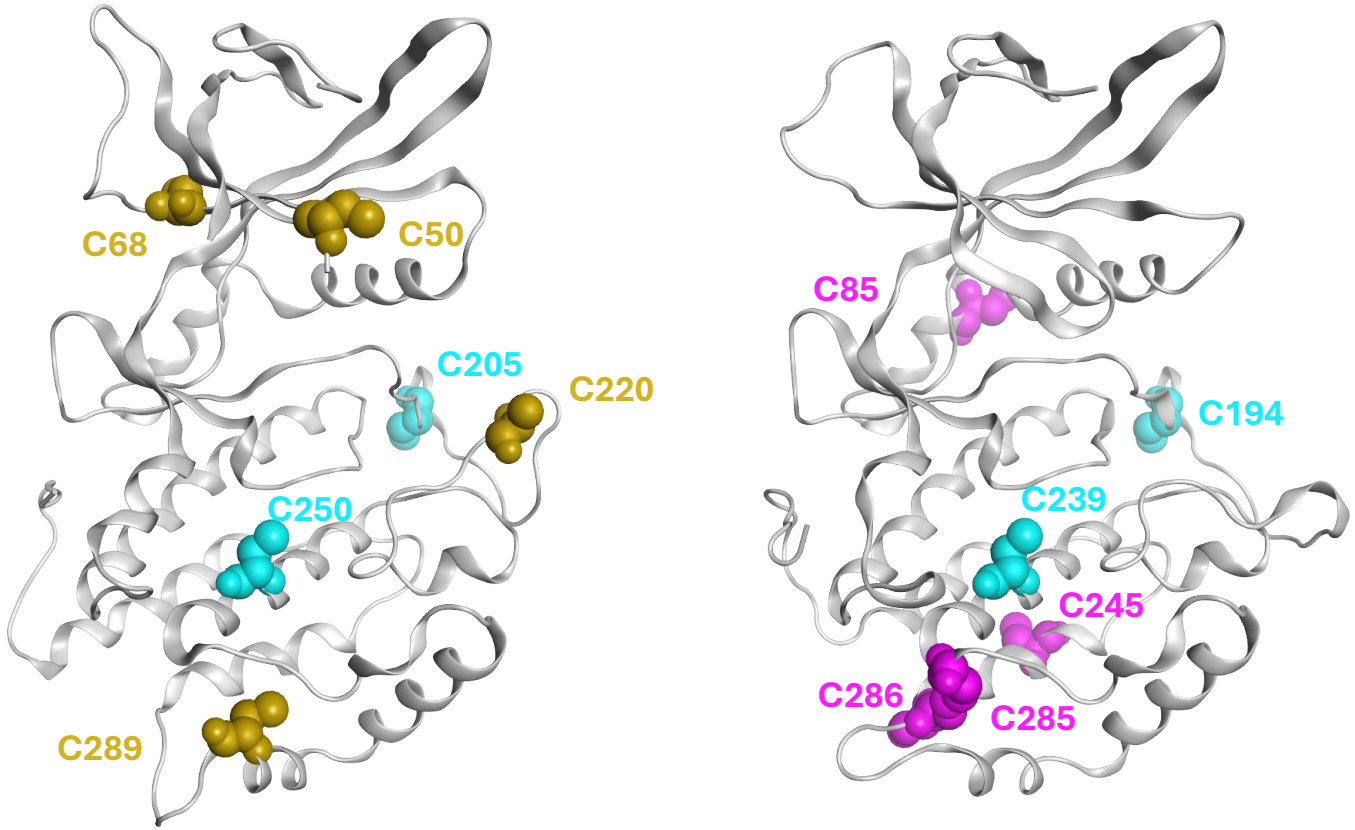
In **VRK2-low cancers** (such as promoter-methylated glioblastomas and neuroblastomas), cell survival becomes entirely dependent on the sister kinase **VRK1**.

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Exploiting unique cysteine residues for covalent, paralog-selective VRK1 inhibition

VRK1	VRK2	Covalent screen

57% sequence identity across catalytic domains



Cys residues unique to VRK1

Cys residues unique to VRK2

Cys residues conserved between VRK1 and VRK2

Library of ~6000 acrylamides

Average MW = 350
90% ≤ 2 HBDs

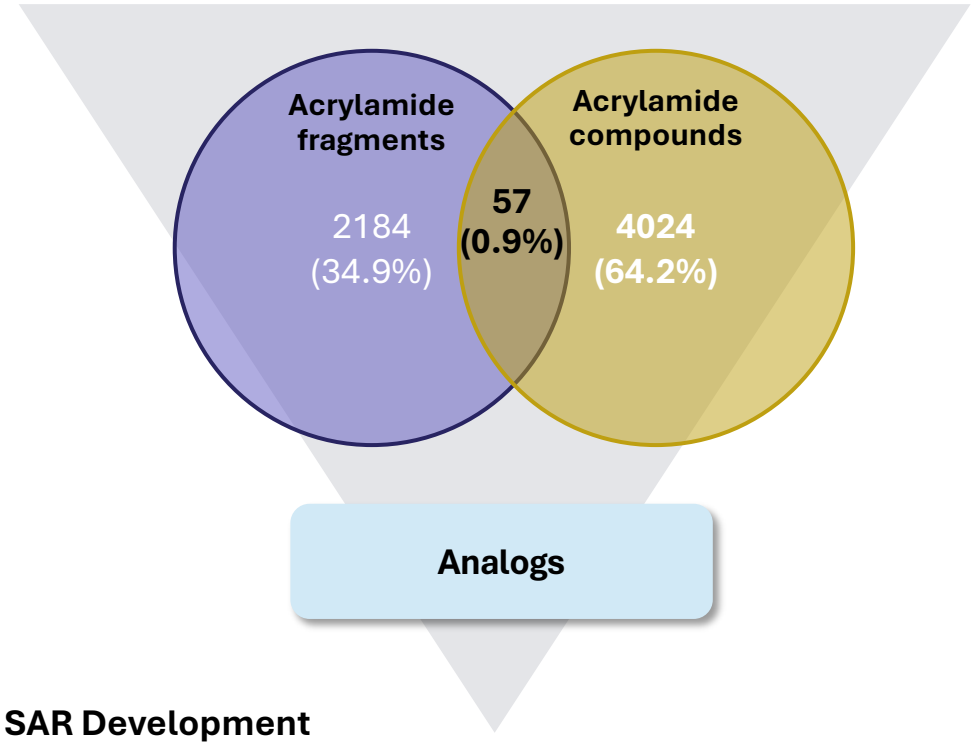
TR-FRET biochemical assay
117 compounds with IC_{50} < 200 μM

MS assay
6 compounds with 70% single adduct formation in VRK1

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Workflow for identification and characterization of covalent VRK1 inhibitors

HTS Screen of Acrylamide Focus Covalent Library in TR-FRET Biochemical Assay



SAR Development

TR-FRET (K_m ATP)
30 min
VRK1

TR-FRET (K_m ATP)
5 hr
VRK1

GSH stability
cmpd

Intact MS
1:1 ratio labeling

MoA study

Select compounds

TR-FRET (Km)
VRK1 mutants C50A,C68A, or a double mutant

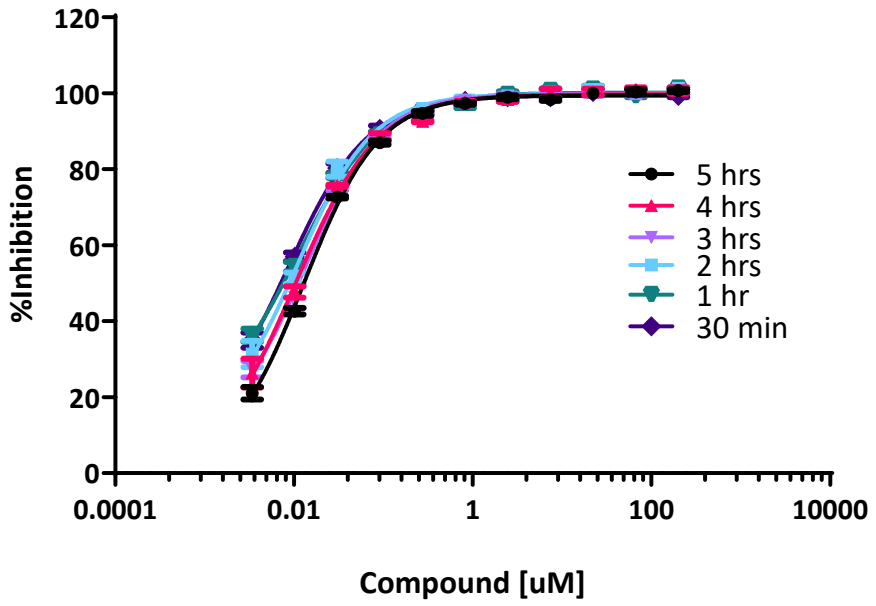
Reversibility assay
VRK1

Intact MS
VRK1 mutants C50A,C68A, or a double mutant

Intact MS
k_{inact}/K_i

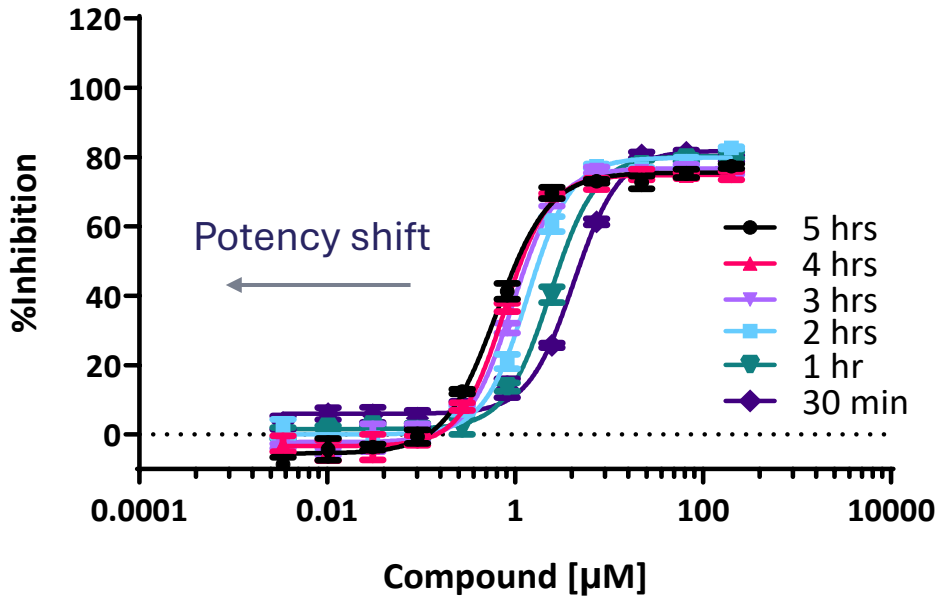
Covalent inhibitors of kinase activity display time-dependent potency increases

TNG Reversible Inhibitors



Incubation of a reversible VRK1 inhibitor shows minimal change in IC_{50} values, indicating time-independent inhibition.

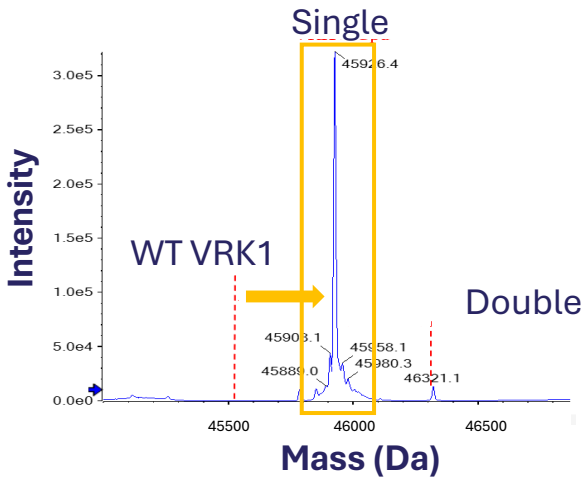
TNG Irreversible inhibitors



A covalent cysteine-targeting inhibitor exhibits a time-dependent leftward shift in IC_{50}, consistent with irreversible binding kinetics.

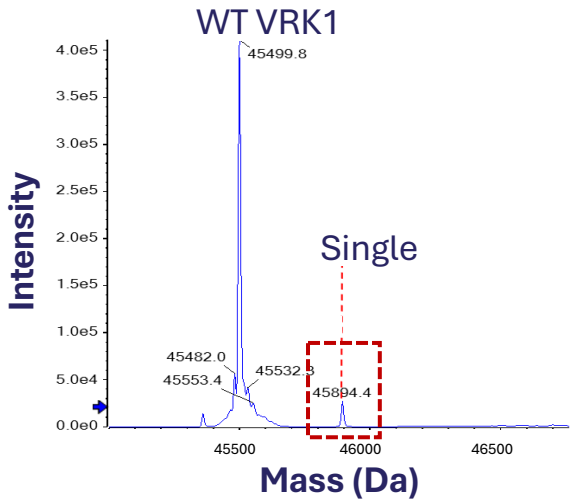
Mass spectrometry and mutational analysis confirms Cys50 as the primary covalent labeling site for VRK1

WT VRK1



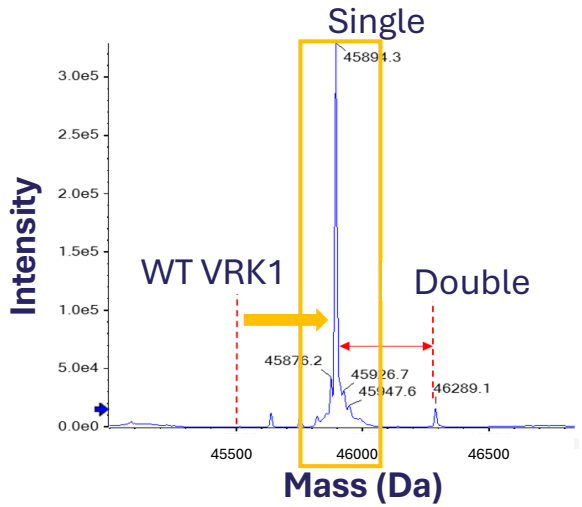
Labeling efficiency (%)	
Single	96.1
Double	3.9

C50A VRK1



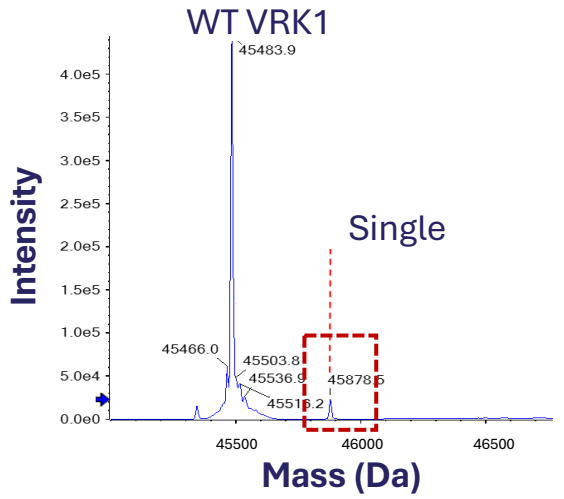
Labeling efficiency (%)	
Single	6.2

C68A VRK1



Labeling efficiency (%)	
Single	95.3
Double	4.7

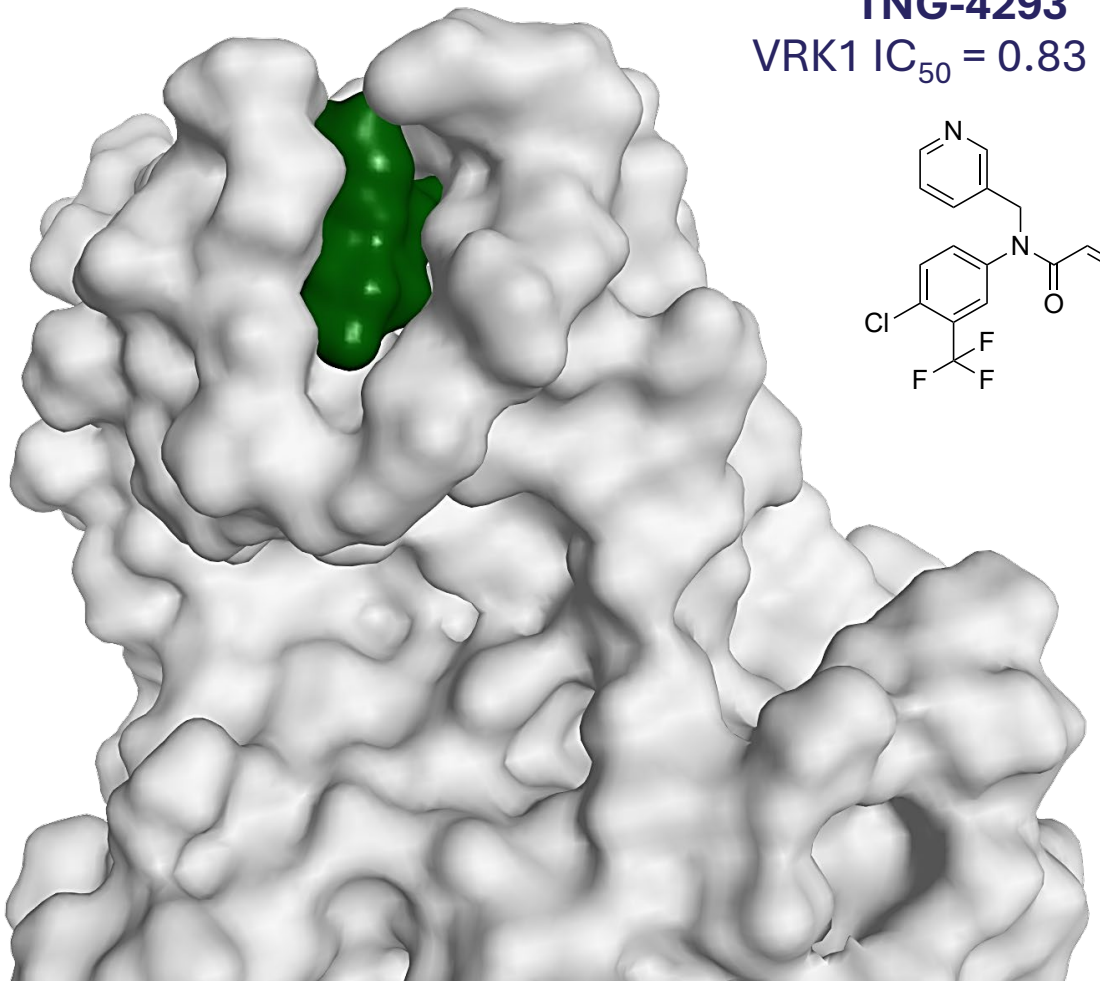
C50A/C68S VRK1



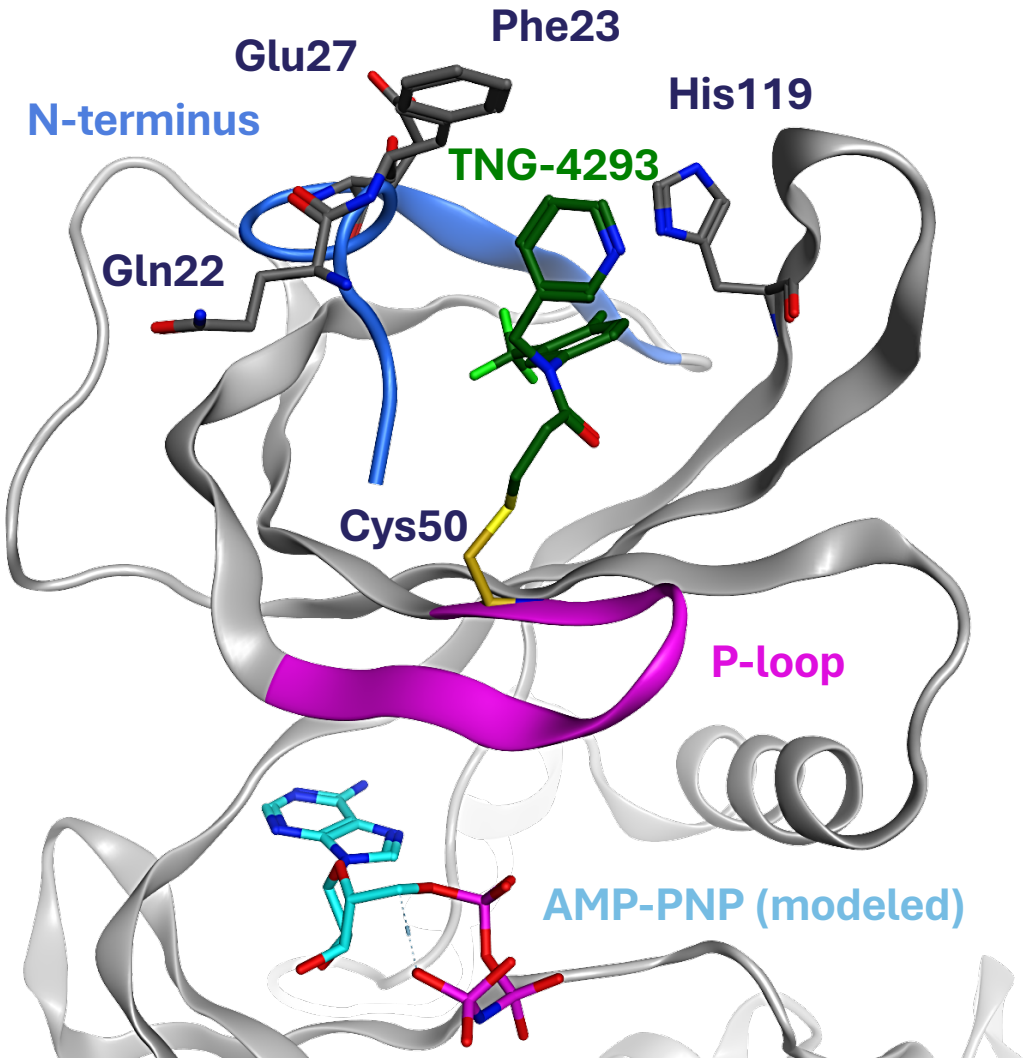
Labeling efficiency (%)	
Single	4.9

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Structural studies of TNG-4293 confirm Cys50 engagement and reveal a novel VRK1 site



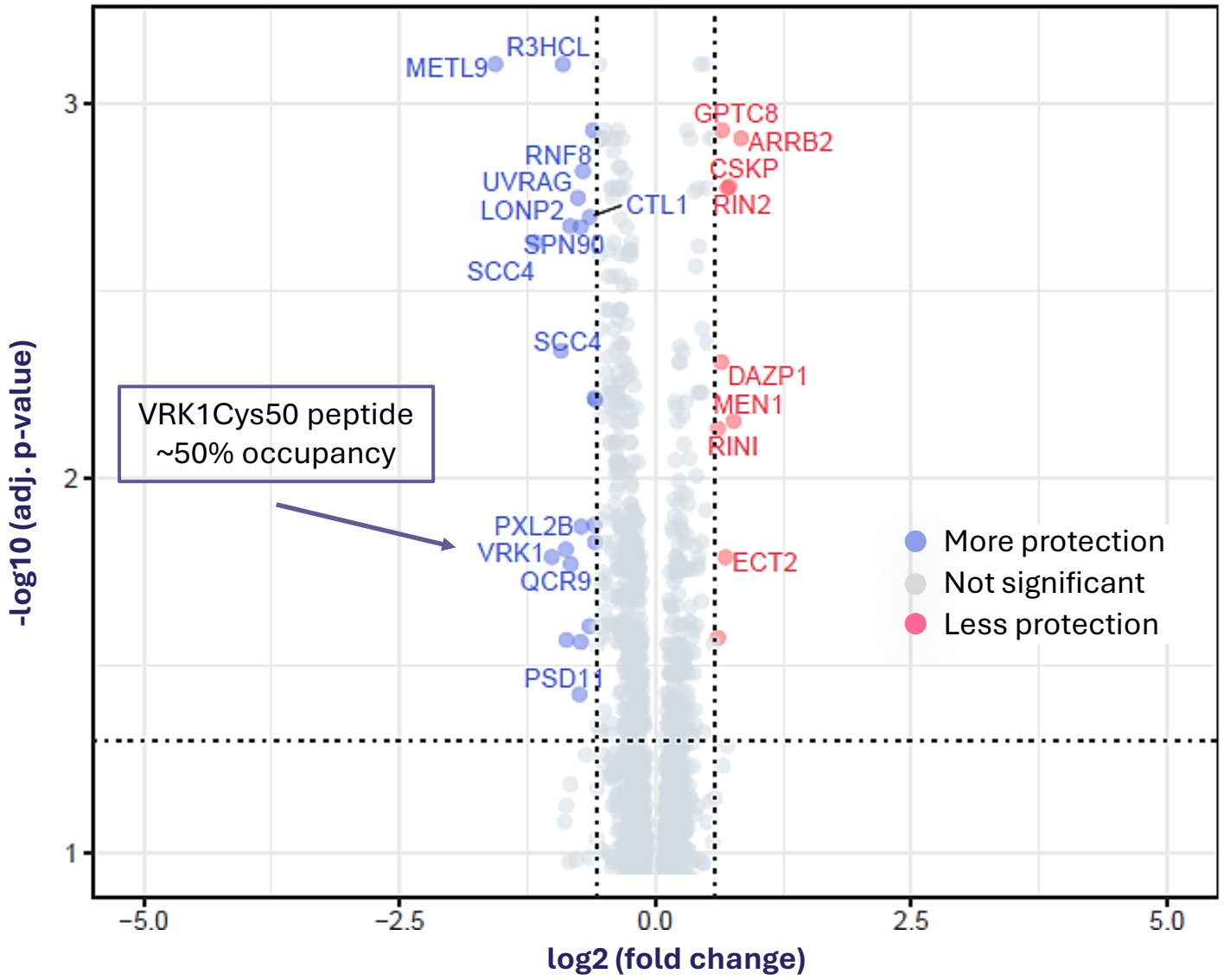
TNG-4293
VRK1 IC_{50} = 0.83 µM



Resolution = 2.1 Å
R_{work} / R_{free} = 0.18 / 0.23

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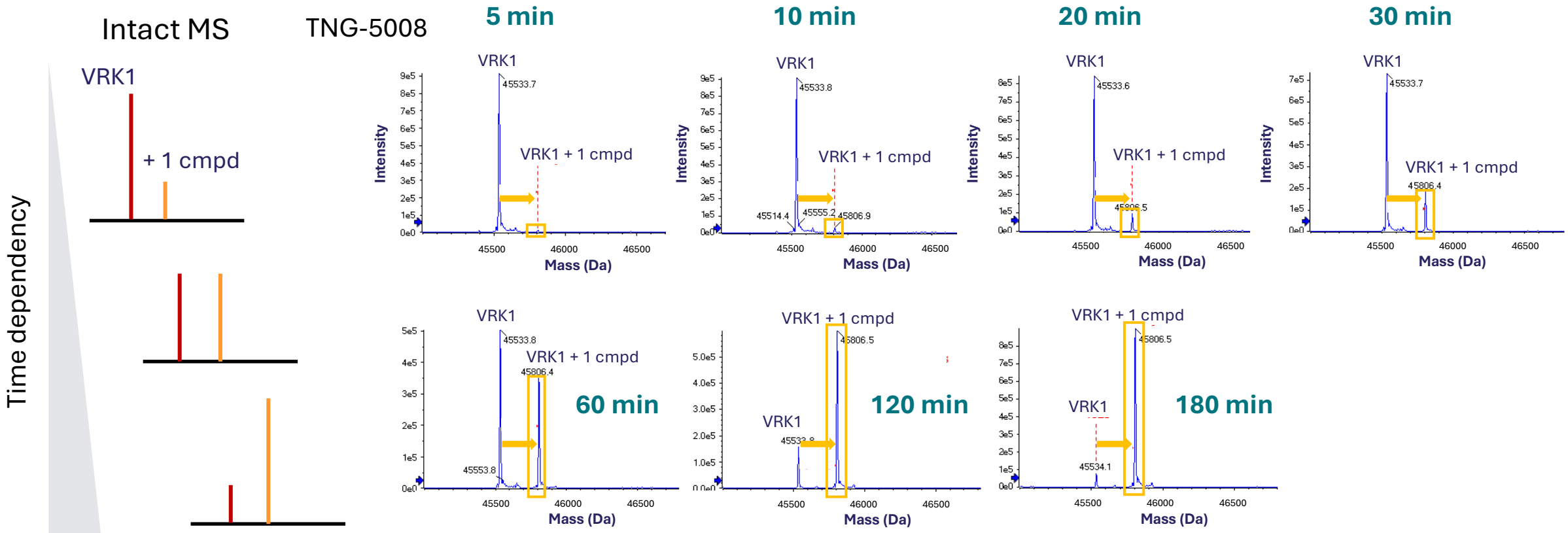
TNG-4293 labels VRK1 in cells but exhibits broad proteome-wide reactivity



Compound: TNG-4293
Cells: U251MG
Digestion Enzyme: Trypsin + Asp-N

Determination of key kinetic parameters for binding (K_i) and reactivity (k_{inact})

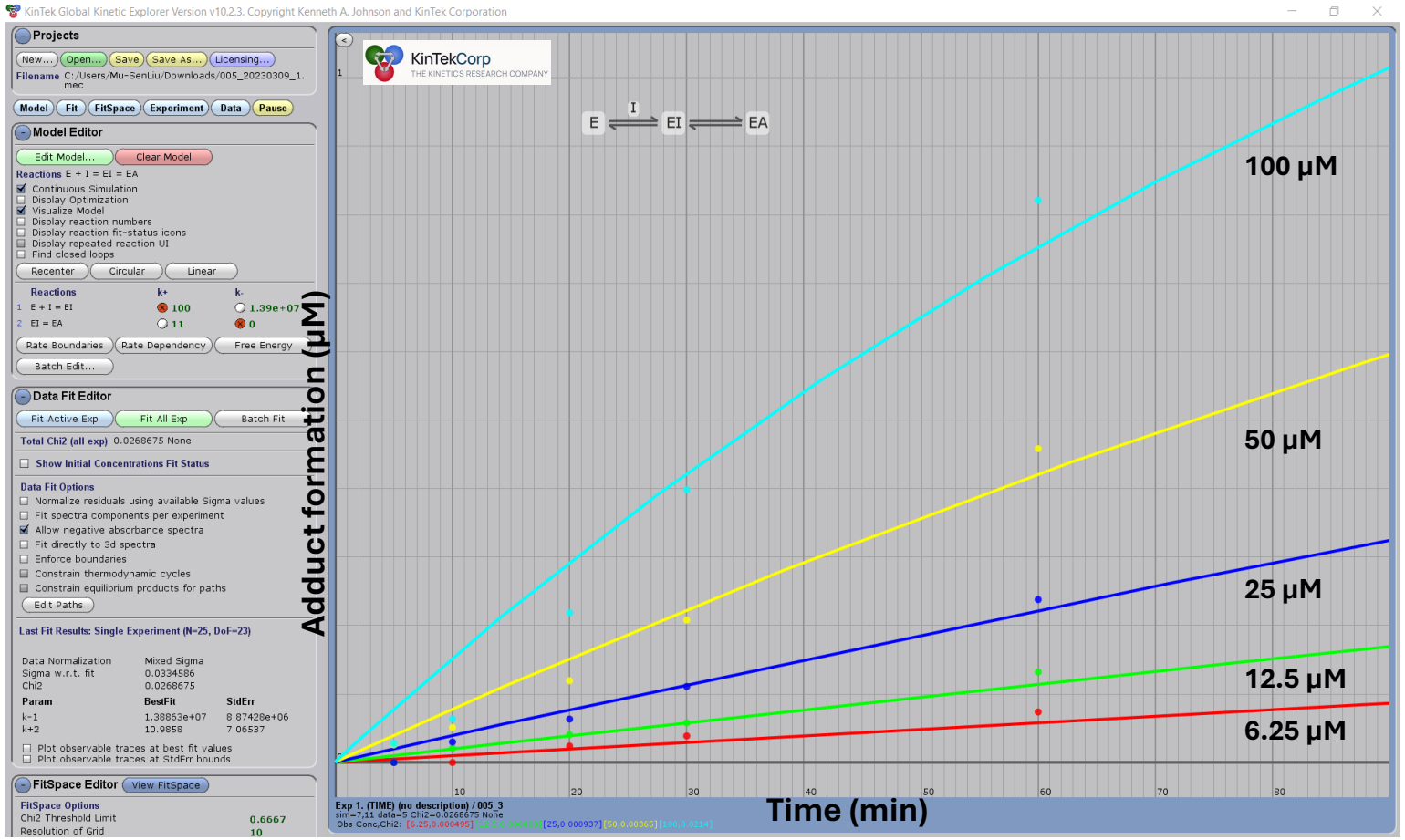
Time-dependent labeling of VRK1 by selected covalent inhibitor



%Adduct Formation = VRK1+ 1 cpd / total VRK1
at 100 µM compound concentration as example

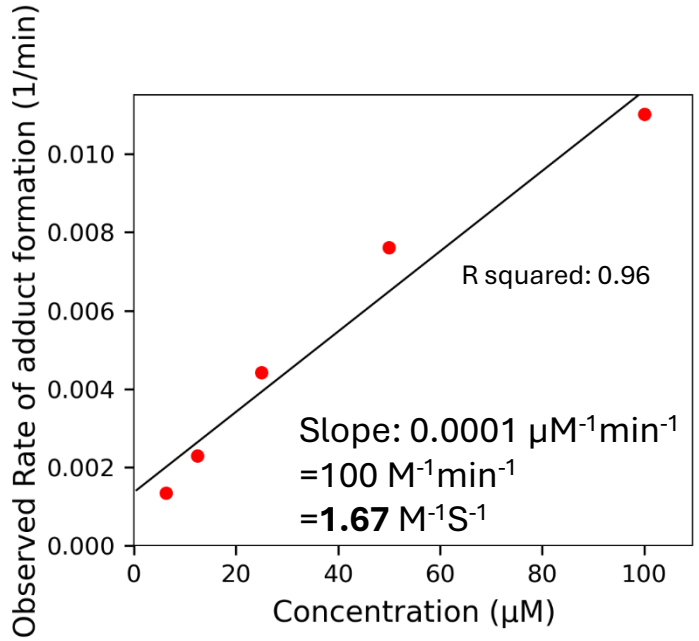
Computational modeling enables data analysis and predictive simulation

To calculate first order rate constant in two steps model by KinTek explorer
E + I = EI = EA (two-step mechanism)



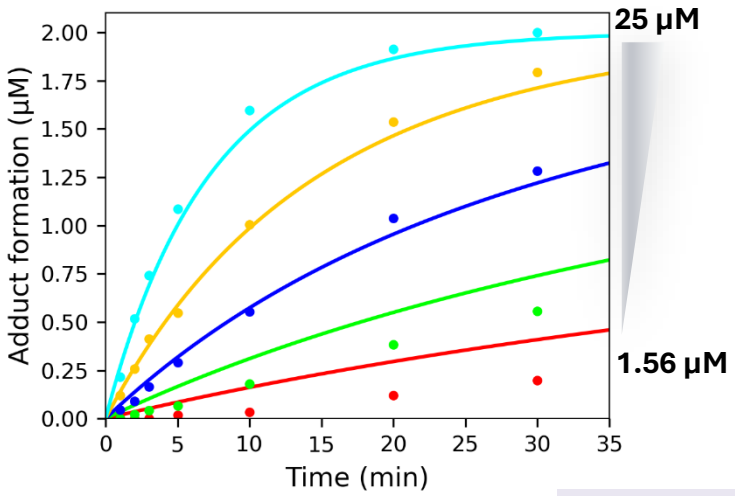
k_{on}= 100 µM^{-1}min^{-1}
k_{off}= 1.39e7 min^{-1}
k_{inact}= 11 min^{-1}= **0.183** s^{-1}
k_i= k_{off}/k_{on} = 1.39e5 µM = **0.139** M
k_{inact}/k_i= 0.183/0.139 M^{-1}s^{-1}= **1.31** M^{-1}S^{-1}

TNG-5008
TR-FRET IC_{50}
30mins/5hours
146.7/41.2 µM



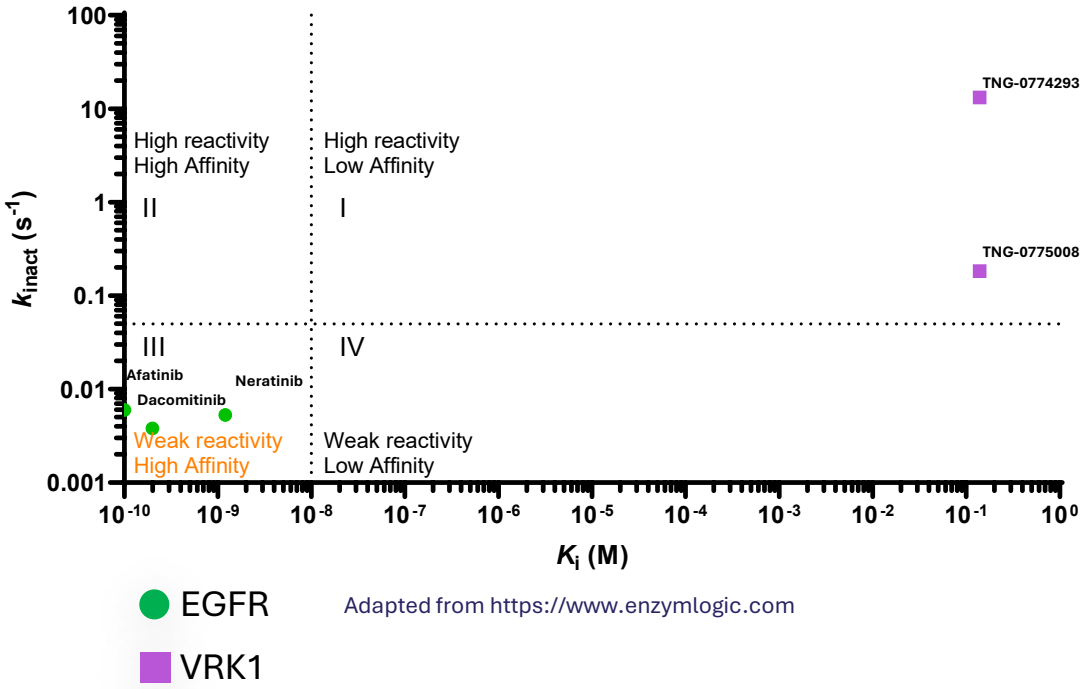
The IC_{50} potency is driven by warhead reactivity (k_{inact}) not binding (K_i)

Time and concentration dependent adduct formation in MS



			Extrapolated	
ID	k_{inact}/K_i ($M^{-1}s^{-1}$)	Biochemical IC_{50} (µM)	k_{inact} (s^{-1})	K_i (mM)
TNG-5008	1.31	41.2	0.183	139
TNG-4293	94.96	0.83	13.2	139

Comparing VRK1 and EGFR covalent inhibitors



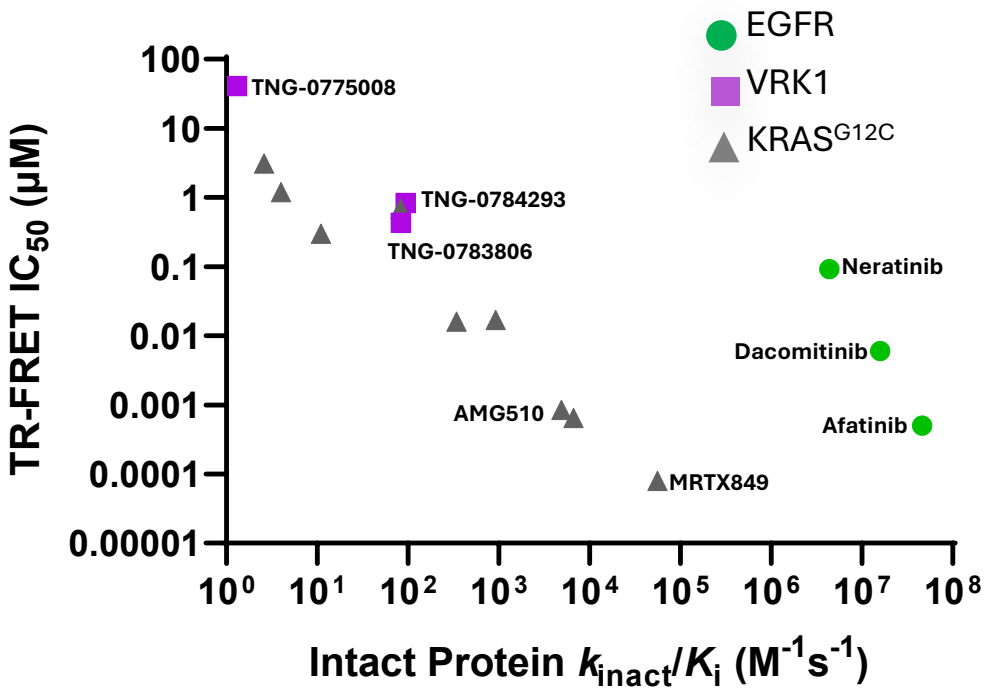
Adapted from https://www.enzymlogic.com

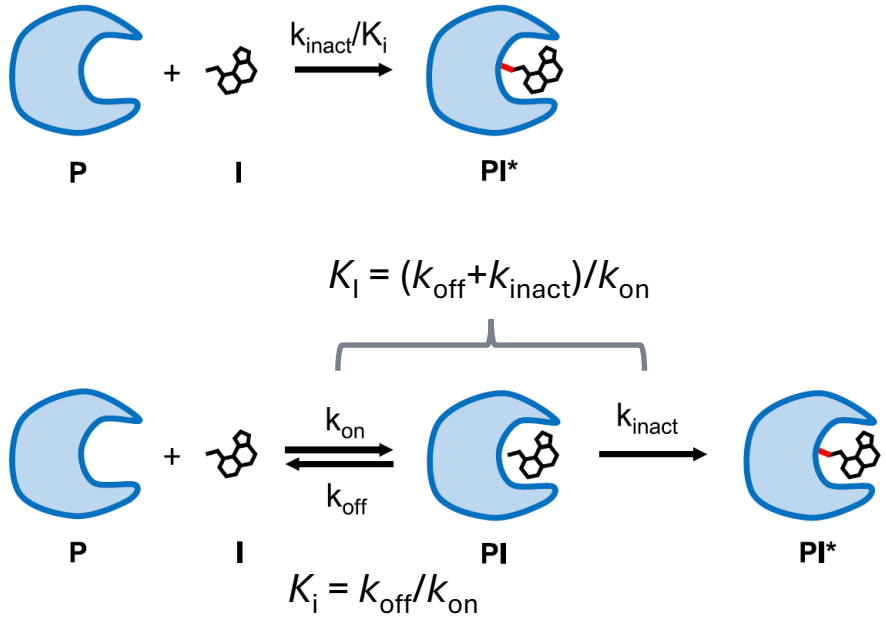
Potency of covalent molecules are mainly driven by k_{inact}

Covalent molecules displayed weak apparent K_i, indicating limiting binding affinity for optimization

Beyond potency: understanding the Mechanism of Action

Covalent inhibitors (acrylamide)





$$P + I \left(\underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PI\right) \underset{k_{-conf}}{\overset{k_{conf}}{\rightleftharpoons}} FI \overset{k_{inact}}{\rightharpoonup} F^*I$$

Biochemical IC_{50} vs k_{inact} / K_i shows an inverse correlation

Adapted from Li, K. S. *et al.* *Analytical Chemistry* **94**, 1230-1239 (2022)

Summary

- Identified selective covalent VRK1 inhibitors through covalent library screening using biochemical TR-FRET and intact protein MS
- Validated Cys50 as the primary site of covalent engagement and a unique structural feature distinguishing VRK1 from VRK2
- Uncovered a previously uncharacterized cryptic binding pocket, expanding opportunities for covalent ligand design
- Established a framework for covalent VRK1 inhibitor discovery, revealing that potency is driven primarily by electrophile reactivity rather than strong non-covalent affinity

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