1O-05

Identification of Covalent JNK Inhibitor

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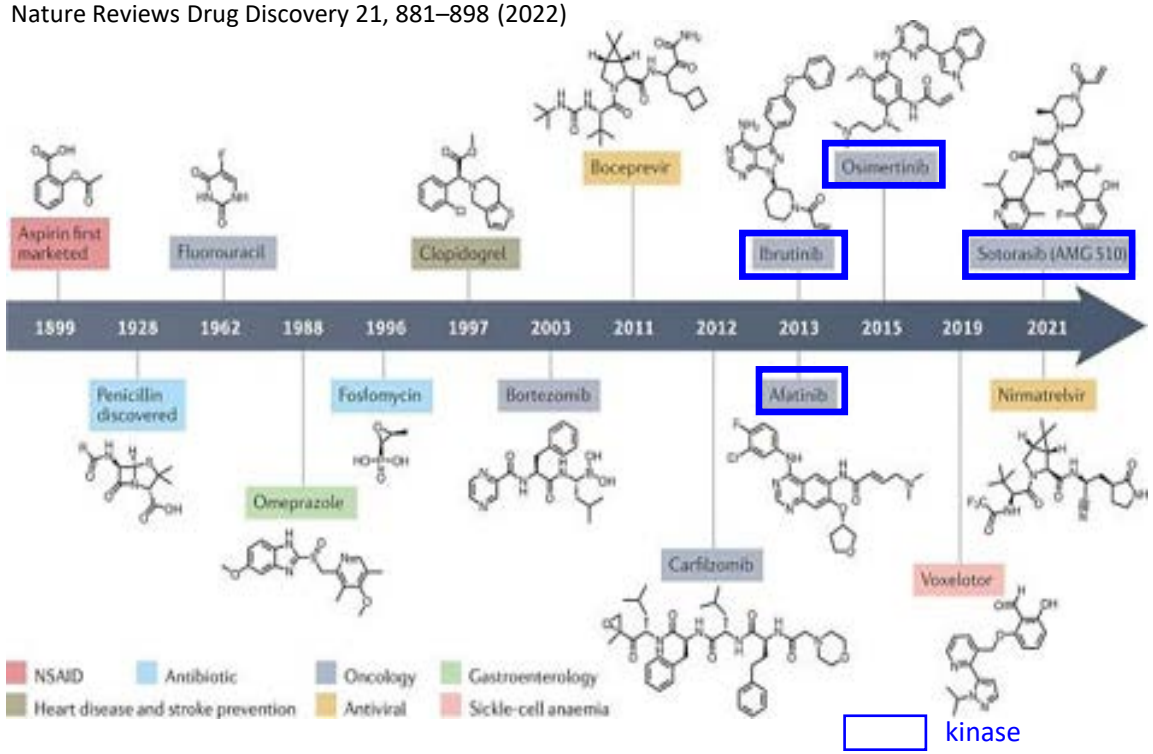
Axcelead Drug Discovery Partners, Inc.

Background

- Rising of covalent inhibitor: targeted EGFR, BTK, JAK etc. covalent kinase inhibitor
- Cysteinome: Ligandable cysteine, relationship with disease
- Screening trend: PROTAC > Degrader > **Covalent inh.** > Reversible inh. > RNA binder

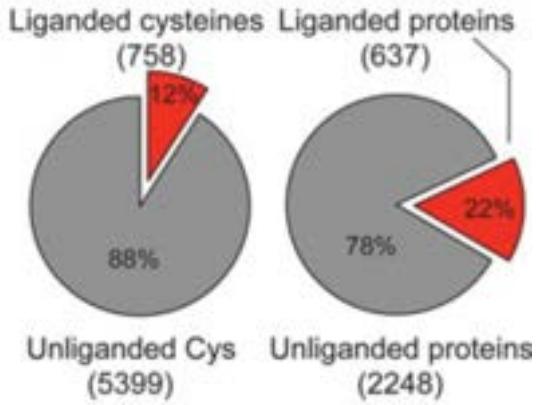
Covalent inhibitor history

Nature Reviews Drug Discovery 21, 881–898 (2022)



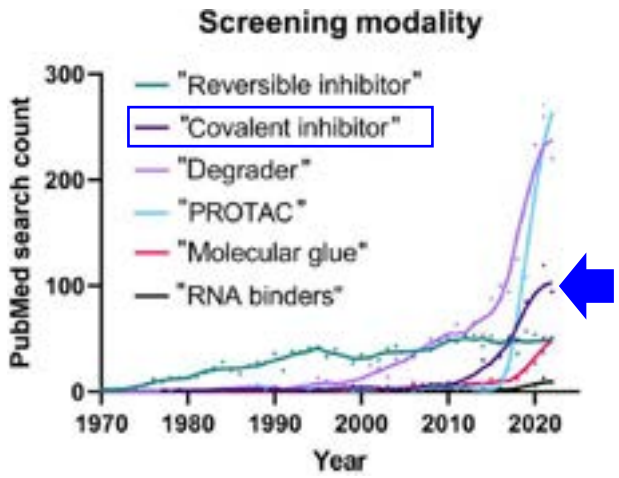
Ligandable cysteine

Nature. 2016, 534, 570–574.



HTS trend

Drug Discovery Today, 28, 2023

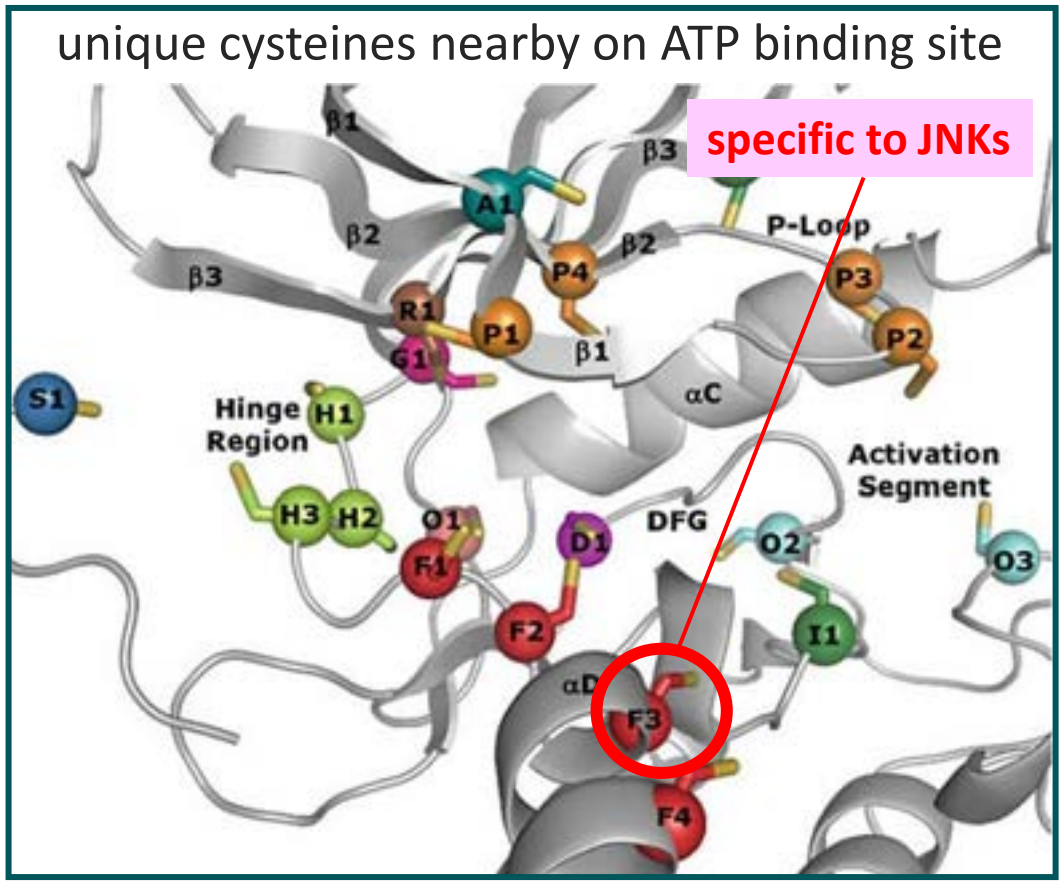


Research on covalent drug is gaining attention.

Concept of Research

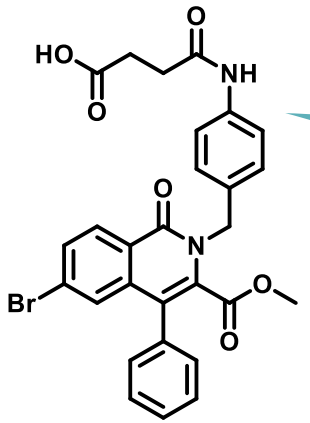
- ✓ c-Jun N-terminal kinases (JNKs) are key signal transducers in the MAPK signaling pathways, involved in inflammation, cancer, and neurodegeneration.

unique cysteines nearby on ATP binding site



Gehringer, M., Covalent kinase inhibitors: an overview. In: Topics in Medicinal Chemistry. Springer, Berlin/Heidelberg, Germany, pp. 1–52, 2020.

previously reported JNK inhibitor*



A

JNK inhibtion IC_{50} = 9.2 nM

potential warhead site search

compound design from SAR

prioritization by docking score

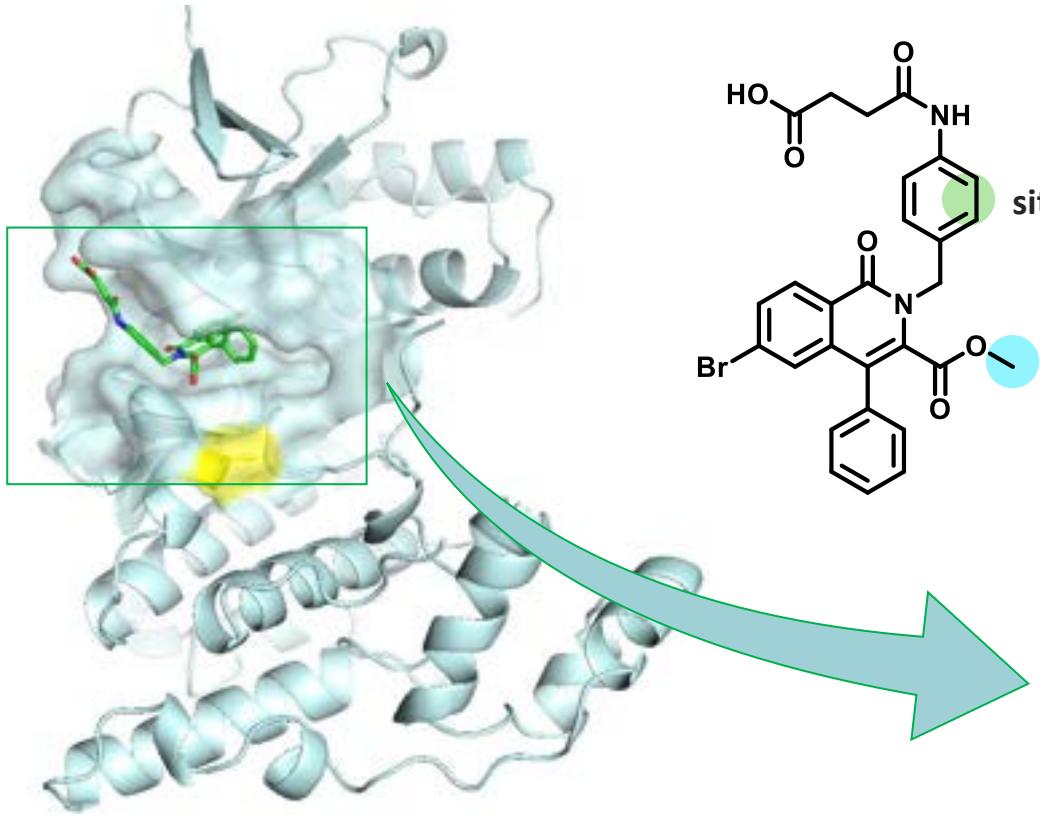
Novel covalent JNK inhibitor

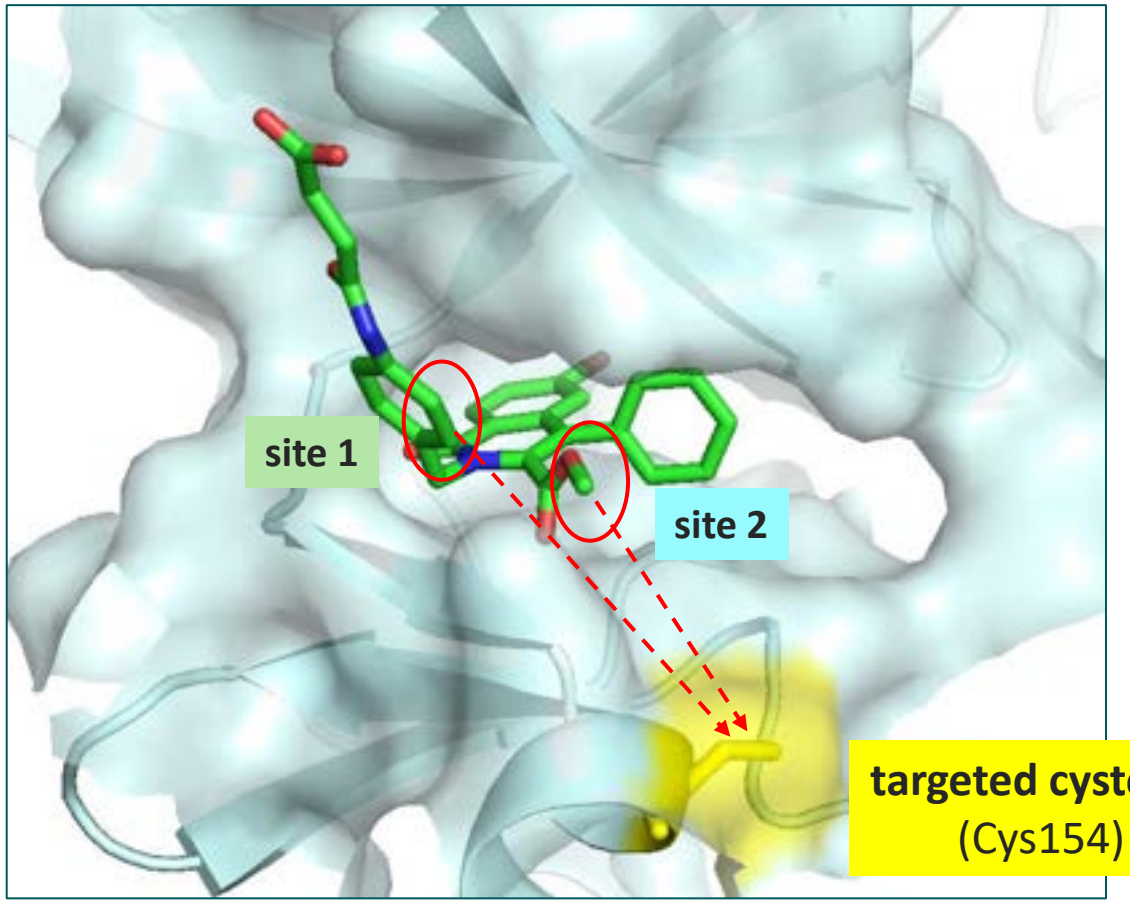
- inhibition activity ⬆
- selectivity ⬆

*) *Bioorg. Med. Chem.* **2008**, *16*, 4715-4732.

Potential warhead site search

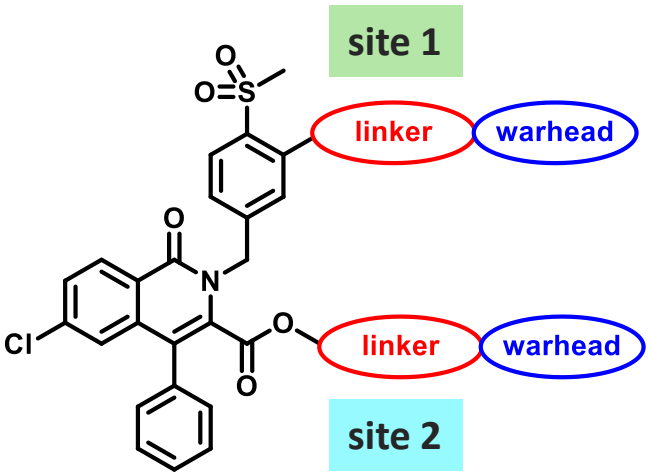
Docking pose of compound **A** to JNK3 protein (PDBID : 2ZDT)





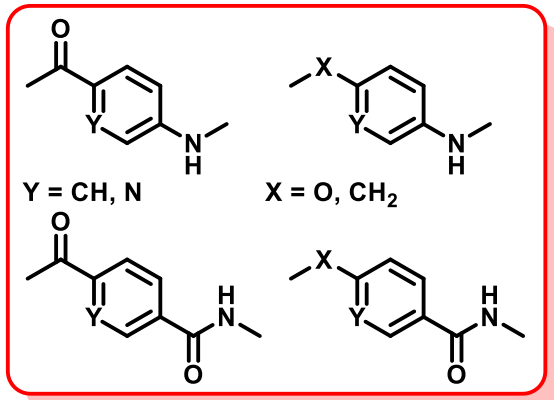
Site 1 and 2 were preferred to introduce warheads.

Compound design: Linkers and Warheads



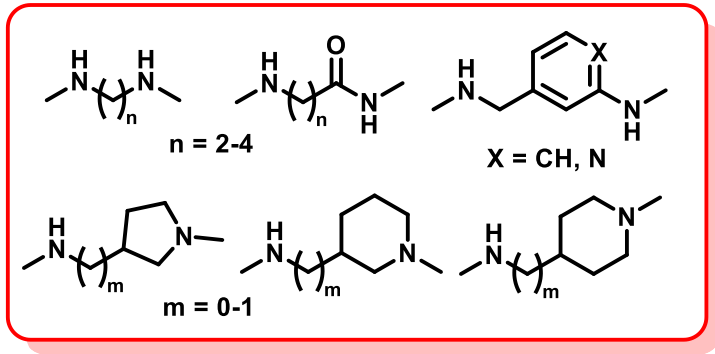
site 1

linkers



site 2

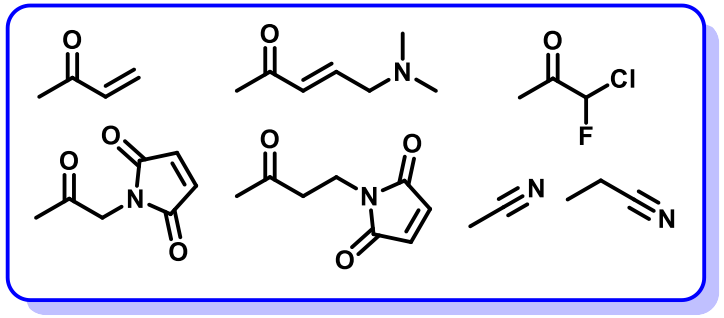
linkers



×

previously reported*) warheads

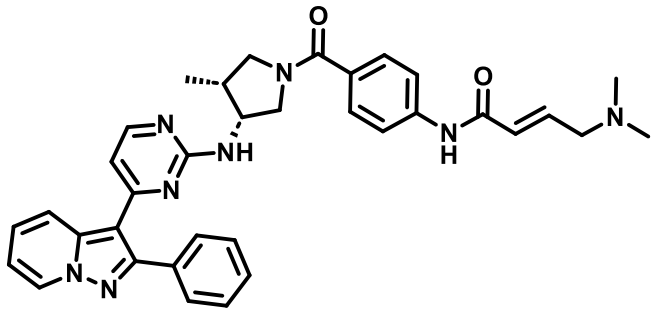
warheads



Narrow down the linker candidates (size, length, stretching direction) from SAR information.

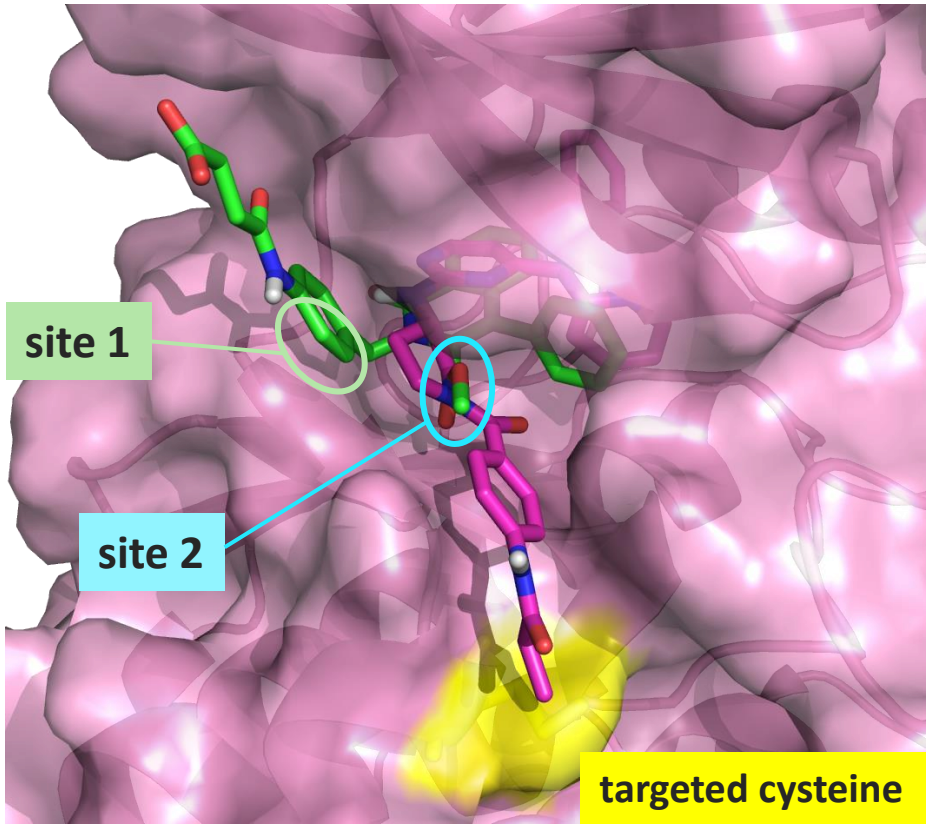
*) *J. Med. Chem.* **2014**, *57*, 10072-10079; *Nat. Chem. Biol.* **2019**, *15*, 250-258; *J. Am. Chem. Soc.* **2013**, *135*, 10582-10585.

Prioritization of compounds to be synthesized

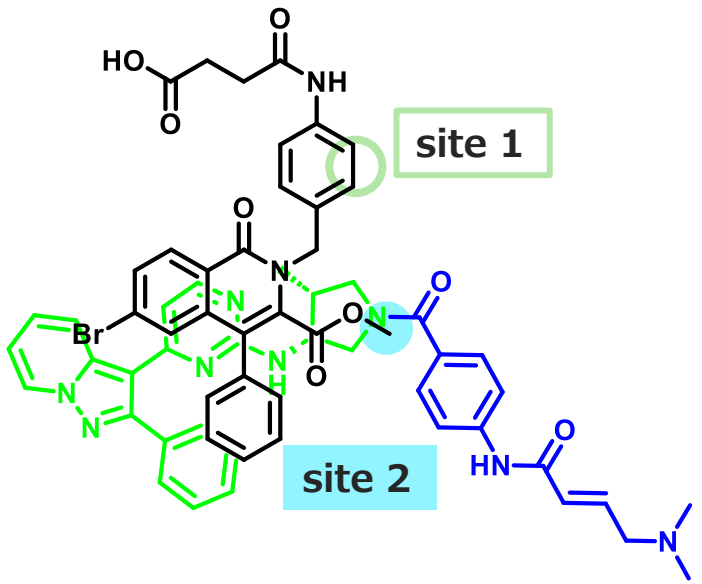


YL5084*
(previously reported covalent JNK inhibitor)

Fig. superposition of **A** and **YL5084**



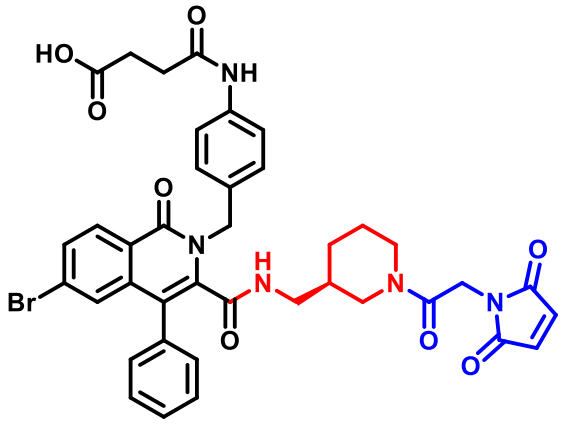
green: compound **A**, magenta: **YL5084**, pink: JNK2 (8ELC)



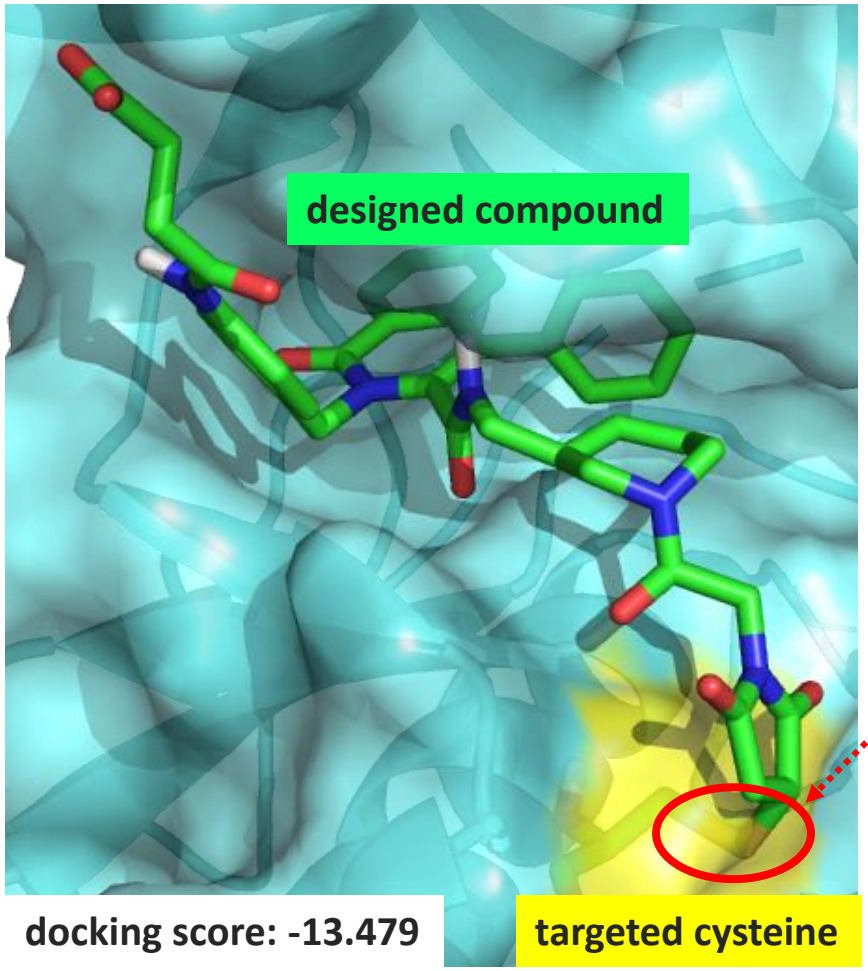
Based on previously reported JNK2 covalent inhibitor, the priority was raised for compounds that extend the linker from site 2.

*) *J. Med. Chem.* **2023**, *66*, 3356-3371.

In silico screening

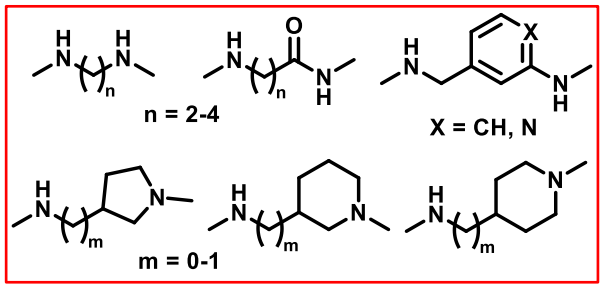


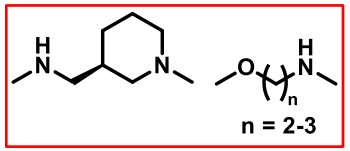
designed compound



docking score: -13.479

covalent bond formation

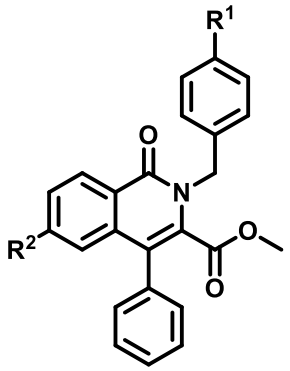




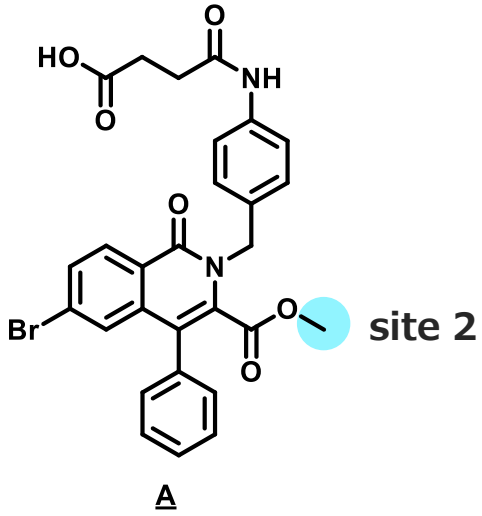
Linkers were prioritized based on **docking score** and **synthetic feasibility**.

Compound design: Modification

Table. SAR information of compound A*)

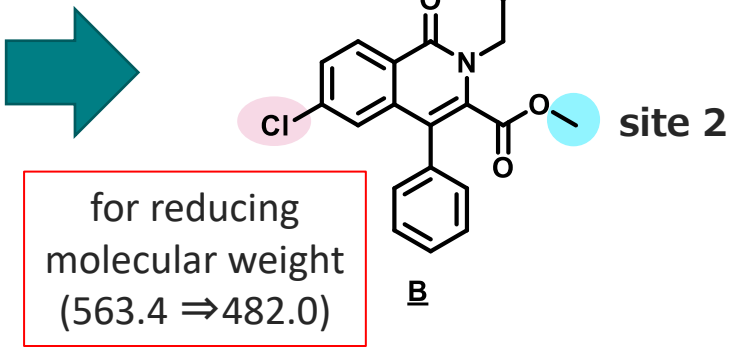


R^1	R^2	JNK1 IC_{50} (nM)
$NHCO(CH_2)_2CO_2H$	Br	9.2
SO_2Me	Br	30
SO_2Me	Cl	28



A

for avoiding β oxidation



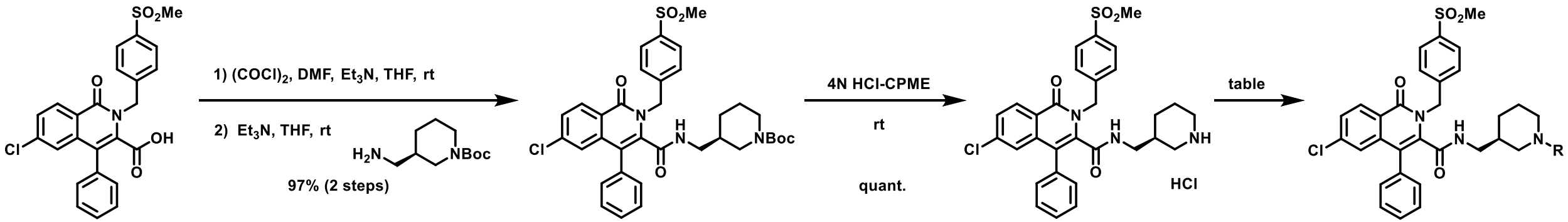
for reducing molecular weight (563.4 ⇒482.0)

B

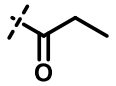
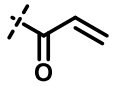
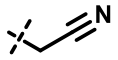
Functional groups which are not related introducing warheads were modified based on SAR information.

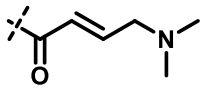
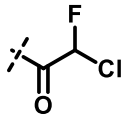
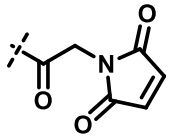
*) *Bioorg. Med. Chem.* **2008**, *16*, 4715-4732.

Synthesis of designed compounds



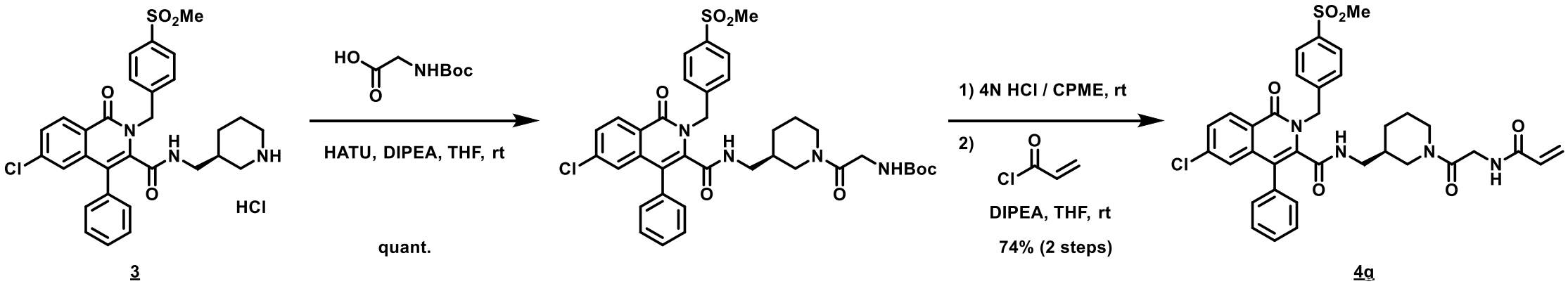
1* 2 3 4

compound	R	Reaction conditions	Yield
4a		DIPEA, THF	77%
4b		DIPEA, THF	72%
4c		K_2CO_3, MeCN	72%

compound	R	Reaction conditions	Yield
4d		HATU, DIPEA, THF	76%
4e		HATU, DIPEA, THF	82%
4f		HATU, DIPEA, THF	93%

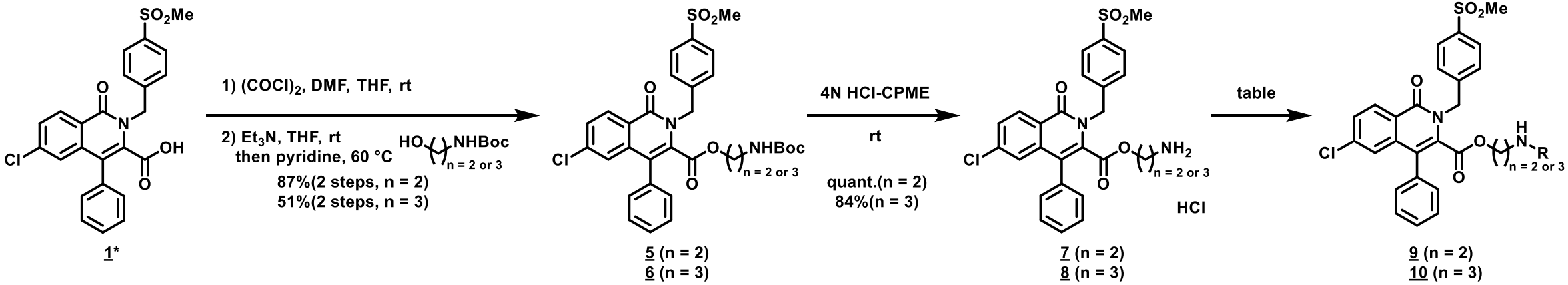
*) *Bioorg. Med. Chem.* **2008**, *16*, 4715-4732.

Synthesis of designed compounds



3 → 4q

Synthesis of designed compounds



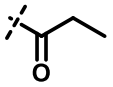
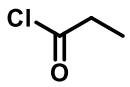
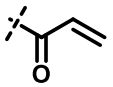
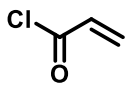
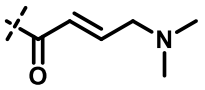
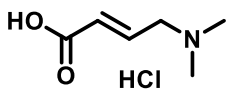
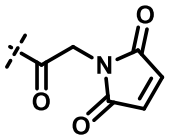
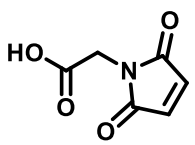
1* (n = 2)

5 (n = 2)
6 (n = 3)

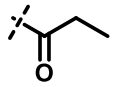
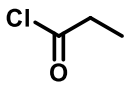
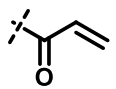
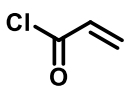
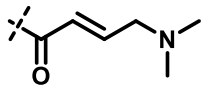
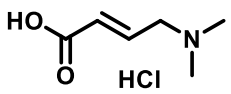
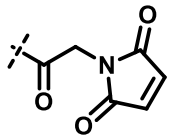
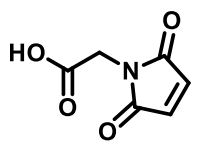
7 (n = 2)
8 (n = 3)

9 (n = 2)
10 (n = 3)

n = 2

compound	R	Reaction conditions	Yield
9a		 DIPEA, THF	84%
9b		 DIPEA, THF	81%
9c		 HATU, DIPEA, THF	61%
9d		 HATU, DIPEA, THF	99%

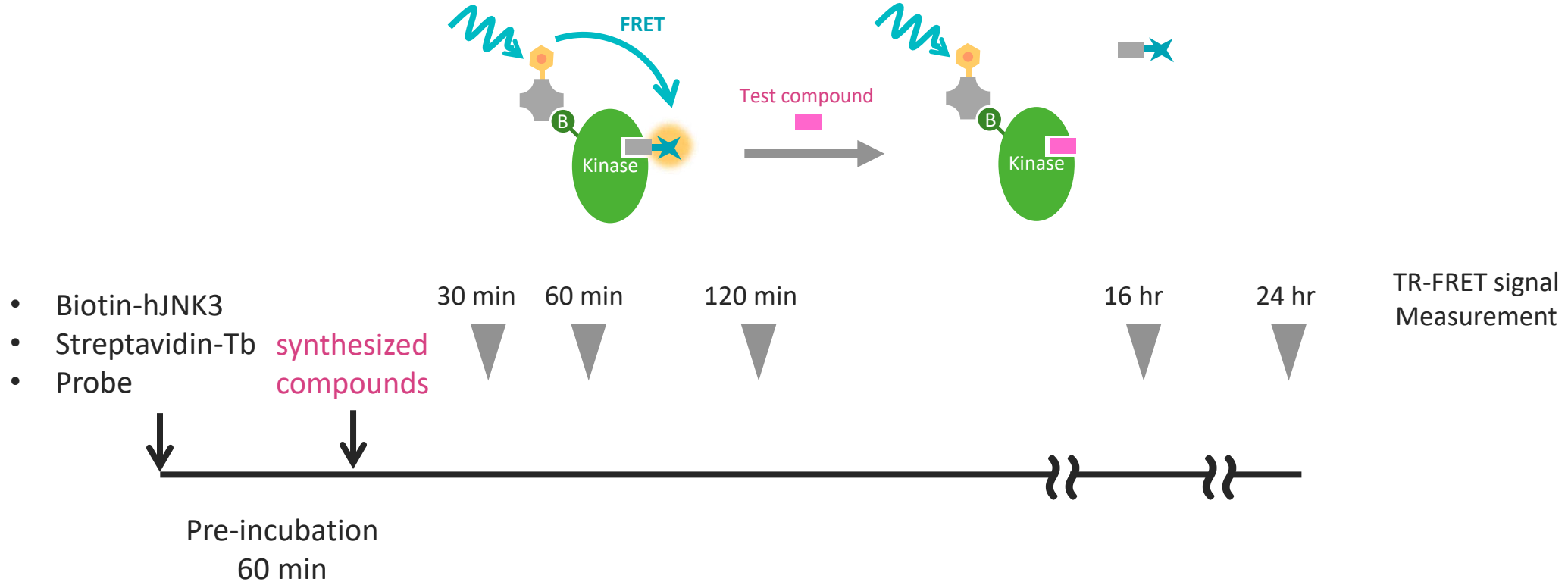
n = 3

compound	R	Reaction conditions	Yield
10a		 DIPEA, THF	87%
10b		 DIPEA, THF	82%
10c		 HATU, DIPEA, THF	92%
10d		 HATU, DIPEA, THF	58%

*) *Bioorg. Med. Chem.* **2008**, *16*, 4715-4732.

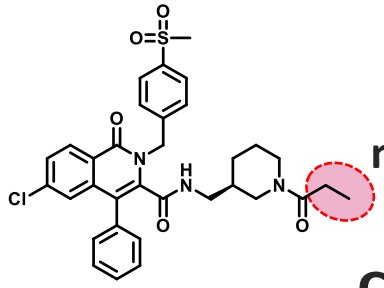
Assessing Time Dependency in Binding Assay : Methods

Probe displacement TR-FRET assay



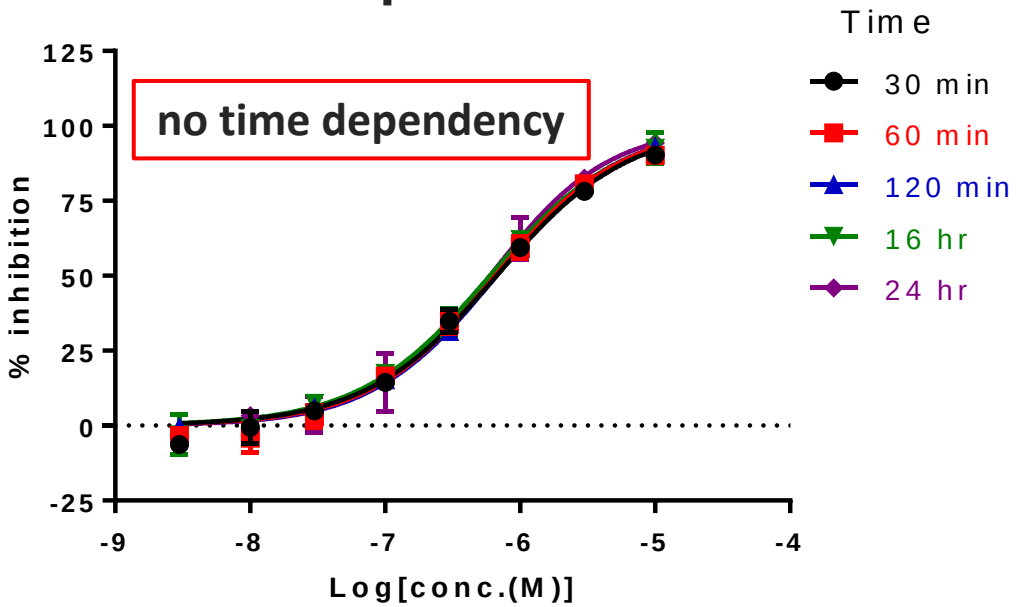
Time dependency of the compound is determined by the increase in inhibition during the incubation time.

Assessing Time Dependency in Binding Assay : Representative Results

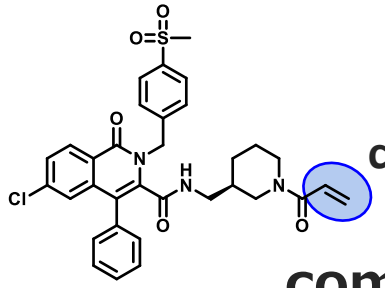


non covalent warhead

compound 4a

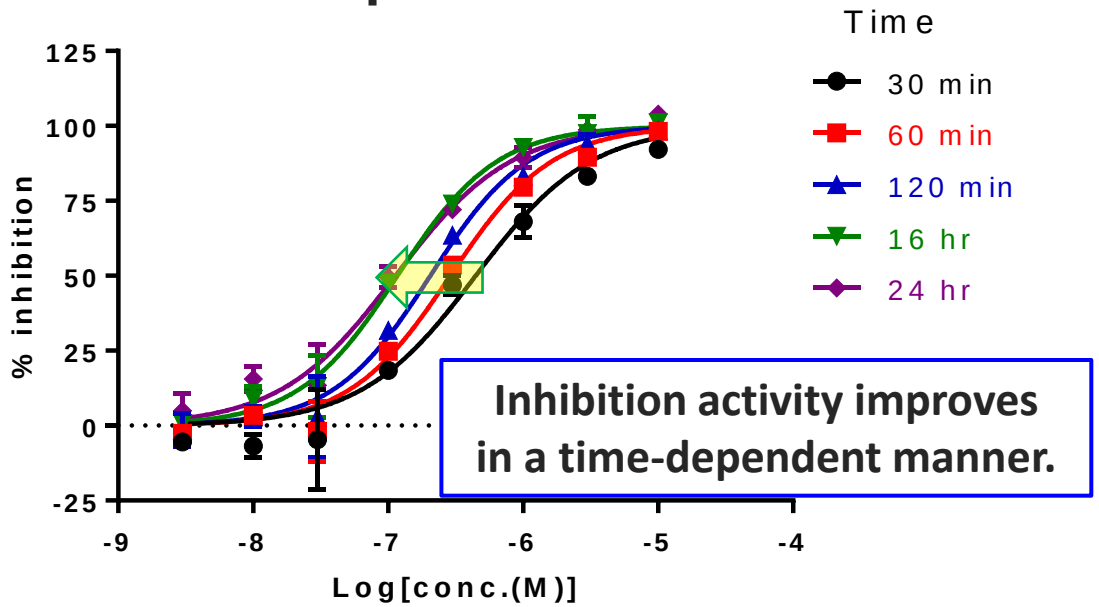


IC_{50} (@60 min) = 655 nM



covalent warhead

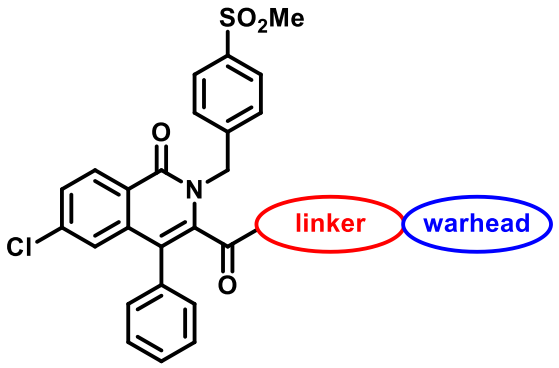
compound 4b

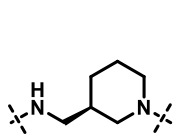
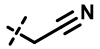
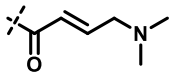
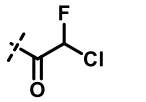
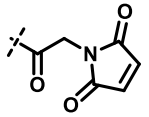
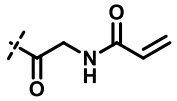


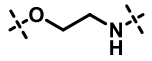
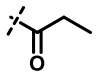
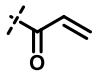
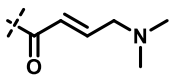
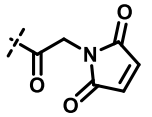
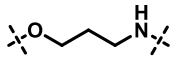
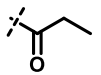
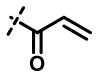
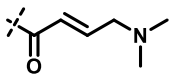
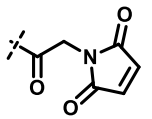
IC_{50} (@60 min) = 289 nM

- ✓ Retained inhibition of JNK3.
- ✓ Compound **4b** inhibited JNK3 in a time-dependent manner.

Assessing Time Dependency in Binding Assay : Overall Results

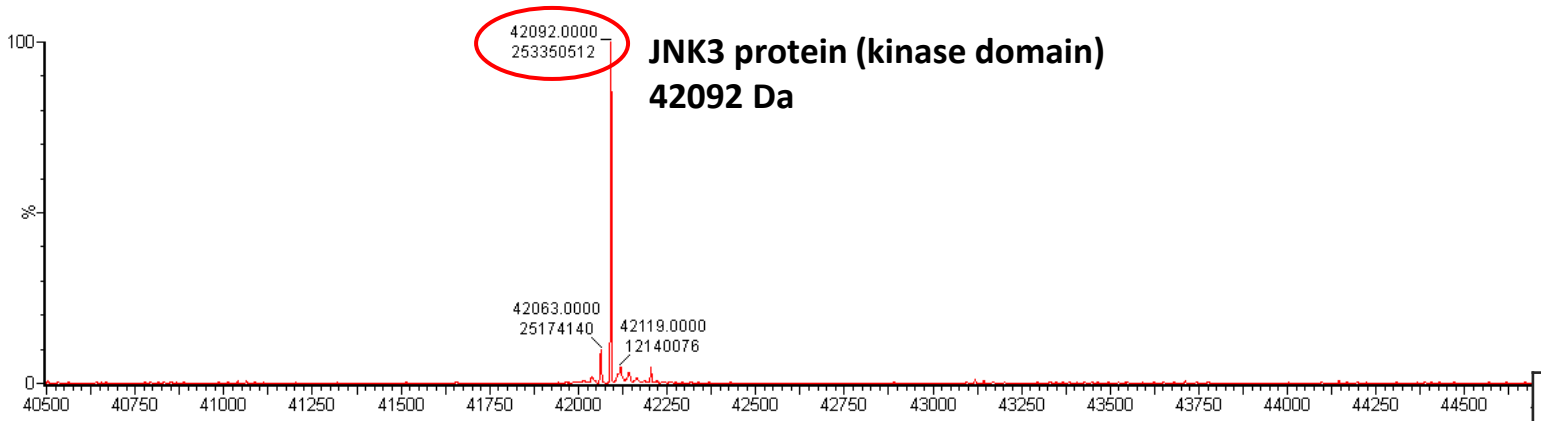


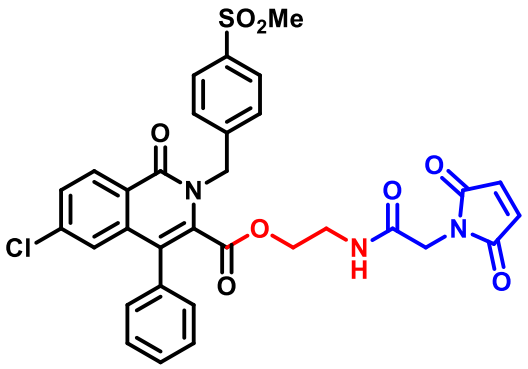
linker	warhead	compound	time dependency	IC_{50} (nM) @ 60 min.
–	–	B	–	28
		4a	–	655
		4b	○	289
		4c	–	553
		4d	–	347
		4e	–	408
		4f	○	0.34
		4g	○	116

linker	warhead	compound	time dependency	IC_{50} (nM) @ 60 min.
		9a	–	196
		9b	–	251
		9c	–	177
		9d	○	0.23
		10a	–	102
		10b	–	102
		10c	○	72
		10d	○	0.14

- ✓ **6 compounds** (**4b**, **4f**, **4g**, **9d**, **10c**, **10d**) inhibited JNK3 in a time-dependent manner.
- ✓ Inhibition activity of **3 compounds** (**4f**, **9d**, **10d**) were improved from compound **A**.

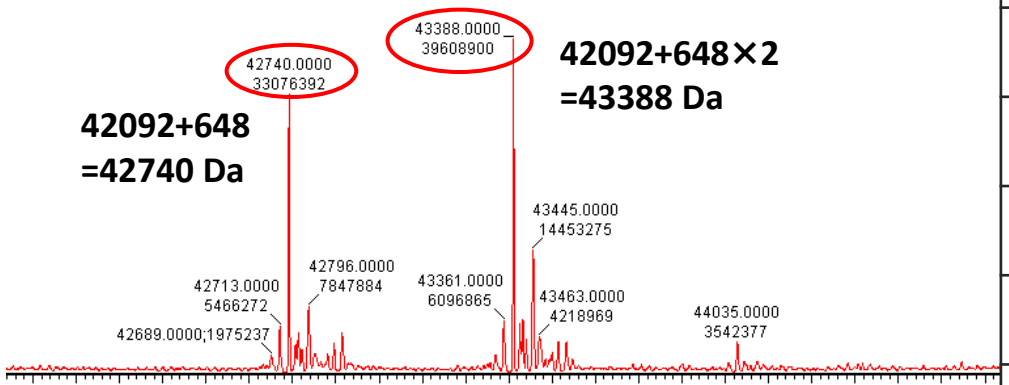
Whole Protein MS of JNK3 + synthesized compounds





Molecular Weight: 648.08

9d



Condition of LC-MS

UPLC: Waters Acquity UPLC I-class

Column: Acquity BEH C4, 300 Å, 1.7µm, 2.1 x 50mmL

Mobile phase A: TFA/ water (0.25/1000)

Mobile phase B: TFA / MeCN (0.25/1000)

MS: Waters Xevo G2-S Tof

Electrospray ionization, Positive ion mode

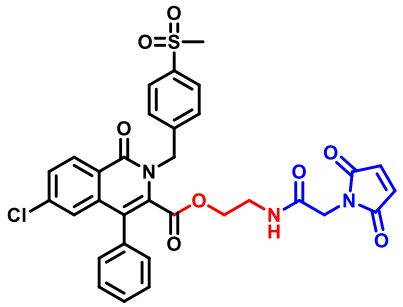
Data analysis: MassLynx, MaxEnt I

comp. ID	Number of adduct	covalent binding
4b	1	single
4f	1,2,3,4	multi
4g	1	single
9d	1,2	multi
10d	1,2	multi

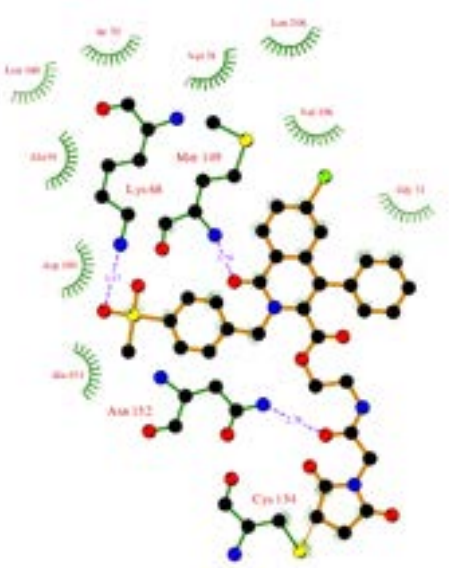
Covalent adduct of **9d** with JNK3 protein was confirmed by protein-MS.

Several compounds confirmed covalent adducts.

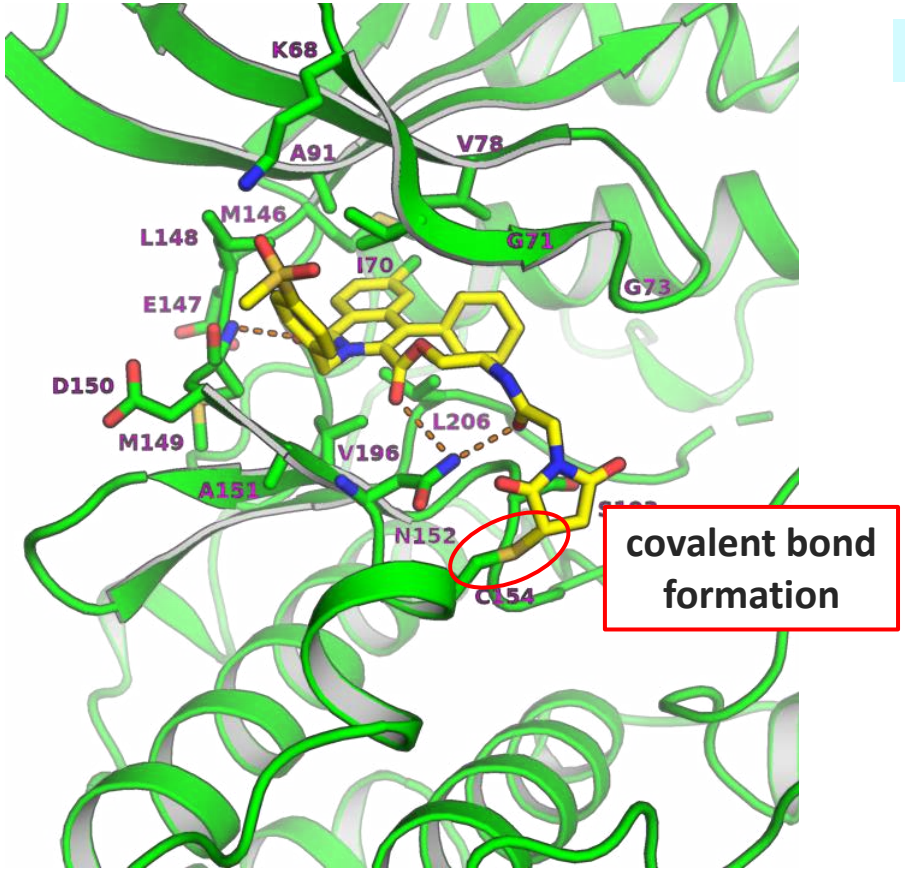
X-ray crystallography



9d



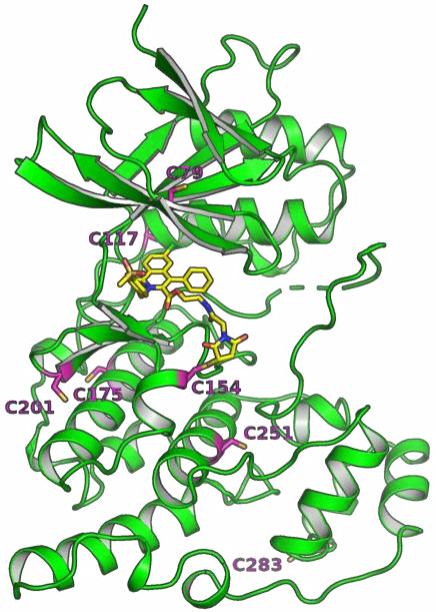
JNK3/comp. 9d (1.7 Å)



Only co-crystals to which **one** compound 9d is bound were obtained.

Mass spectroscopy indicated **9d** is bound to a few sites.

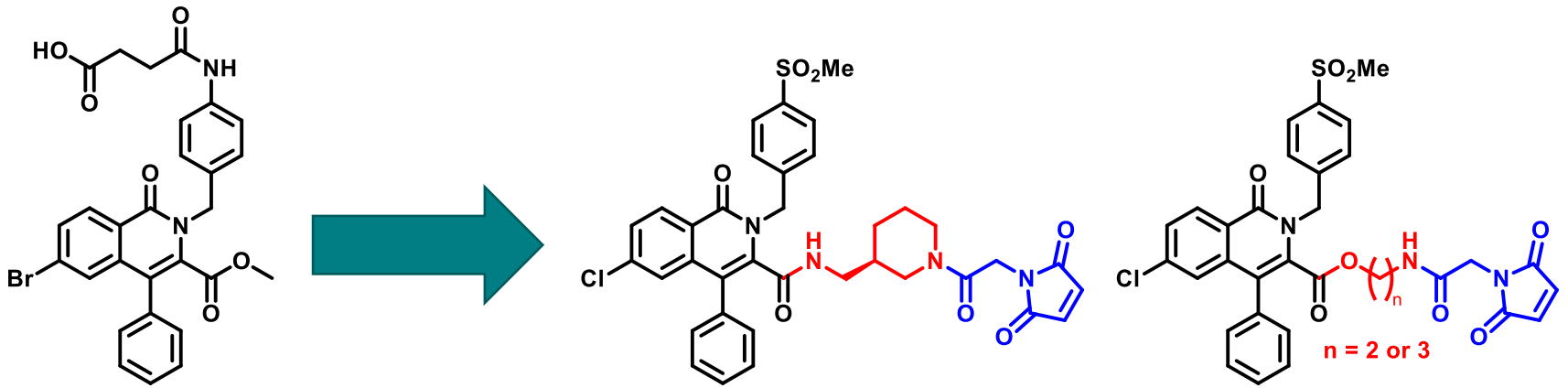
The potential covalent modification sites



The kinase domain contains **7** cysteines.

Covalent binding to the expected cysteine residue (C154) was confirmed, but the additional modification sites remain unidentified.

Summary



JNK3 IC_{50} 0.34 nM (@ 60min.)

JNK3 IC_{50} 0.23 nM (n = 2, @ 60min.)
JNK3 IC_{50} 0.14 nM (n = 3, @ 60min.)

- ✓ Designed compounds **retained inhibition of JNK3**.
- ✓ Several compounds inhibited JNK3 **in a time-dependent manner**.
- ✓ **Adducts of designed compound with JNK3 protein** was confirmed by Protein-MS.
- ✓ **Covalent binding to the expected cysteine** was confirmed by X-ray crystallography.

Tasks to do in future

- Selectivity assay (JNK3/1 and 2)
- Site identification
- *In vivo* assay

Thank you !

Access to Accelerate Lead for Drug Discovery

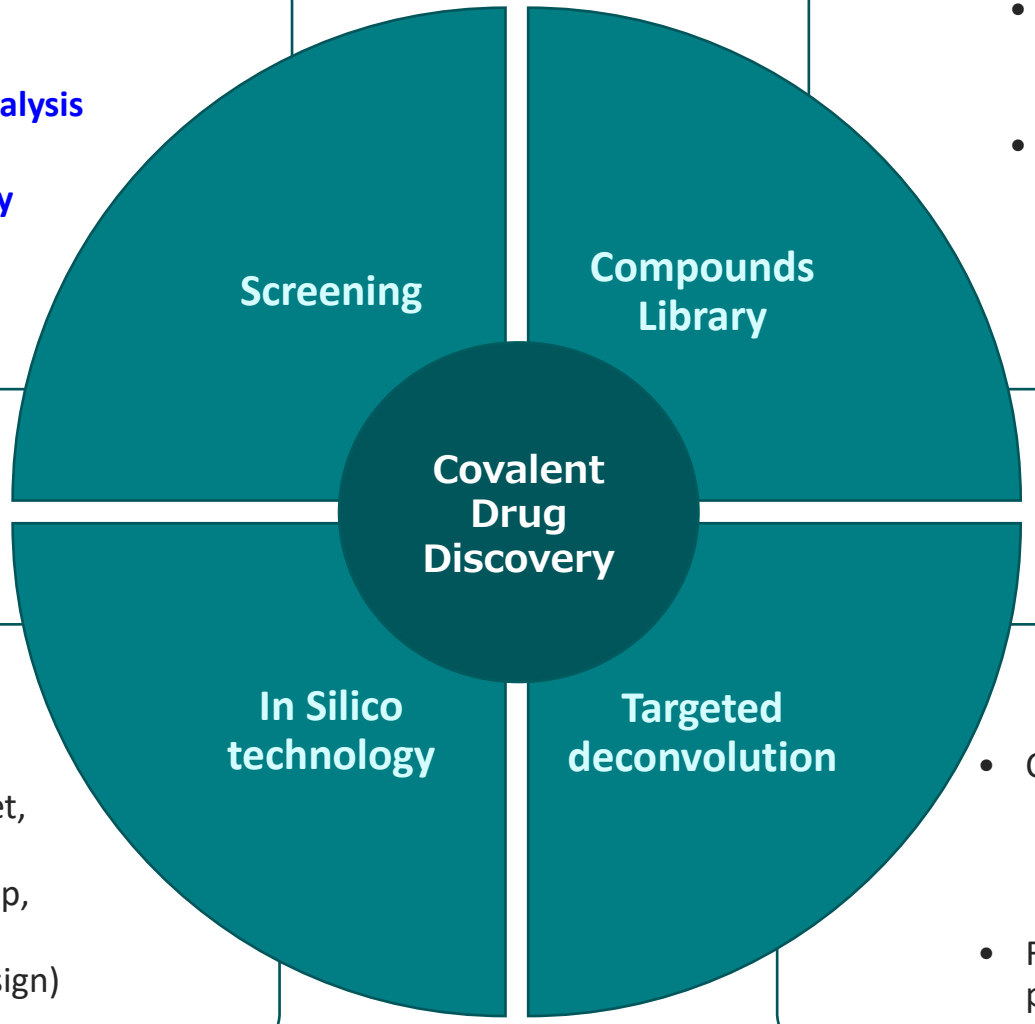
Axcelead Drug Discovery Partners, Inc.

https://axcelead-us.com/

https://www.axcelead.com/

appendix

ADDP capability for covalent drug discovery



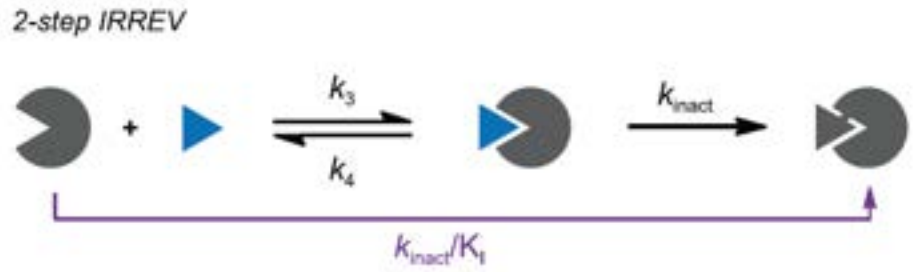
- Enzyme assay
- Cell assay
- **Compound evaluation analysis**
  - **Kinact/Ki**
  - **Time dependency**
  - general reactivity
- **Crystallography**
- **Whole Protein MS assay**

- Cysteine focused covalent library (2,000 compounds)
- Diversity library (500,000 compounds)

- **Targeted protein analysis** (Amino acid search, Pocket, LigMap)
- **Compound design** (LigMap, **covalent docking**)
- FBDD (MMPBSA, AutoDesign)

- Chemical proteomics
  - Protein identification
  - Site identification
  - Photo affinity labelling
- Finger printing (target or pathway prediction)

Reaction kinetic analysis of covalent inhibitor-1



Inactivation Potency

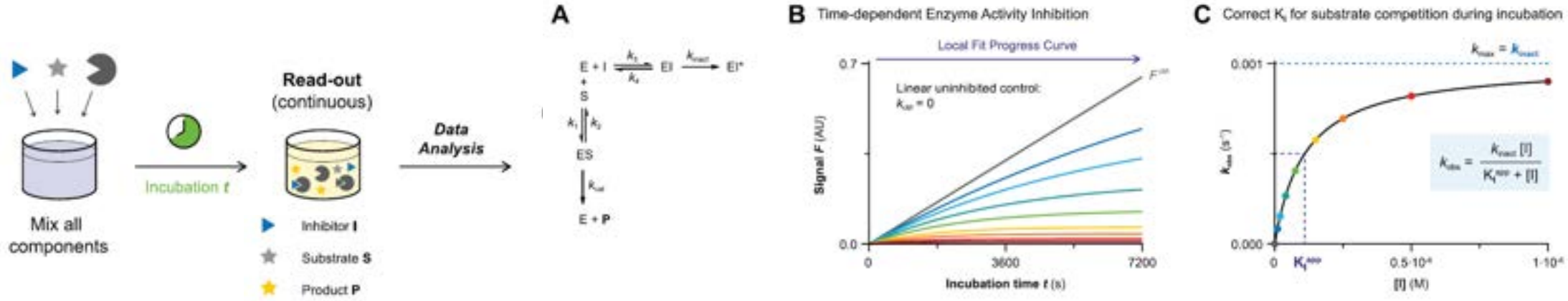
$$k_{inact}/K_I = \frac{k_3\, k_{inact}}{k_4 + k_{inact}}$$

Inactivation Constant

$$K_I = \frac{[E]\,[I]}{[EI]} = K_i + \frac{k_{inact}}{k_3}$$

Covalent Inhibitor Binding Kinetics

$$F_t = v_s t + \frac{v_i - v_s}{k_{obs}}\left[1 - e^{-k_{obs}t}\right] + F_0$$



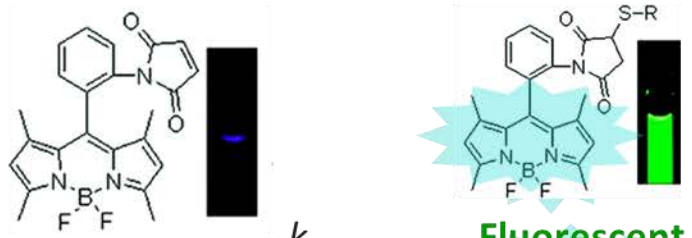
Progress curve analysis for two-step irreversible covalent inhibition.

Curr Protoc. 2022 Jun;2(6)

Inhibitor concentration–dependent K_{obs} reaches K_{inact} at saturating inhibitor concentration ($K_{max} = K_{inact}$).
Half-maximum K_{obs} = ½K_{inact} is reached when inhibitor concentration equals the apparent inactivation constant K_I^{app}.

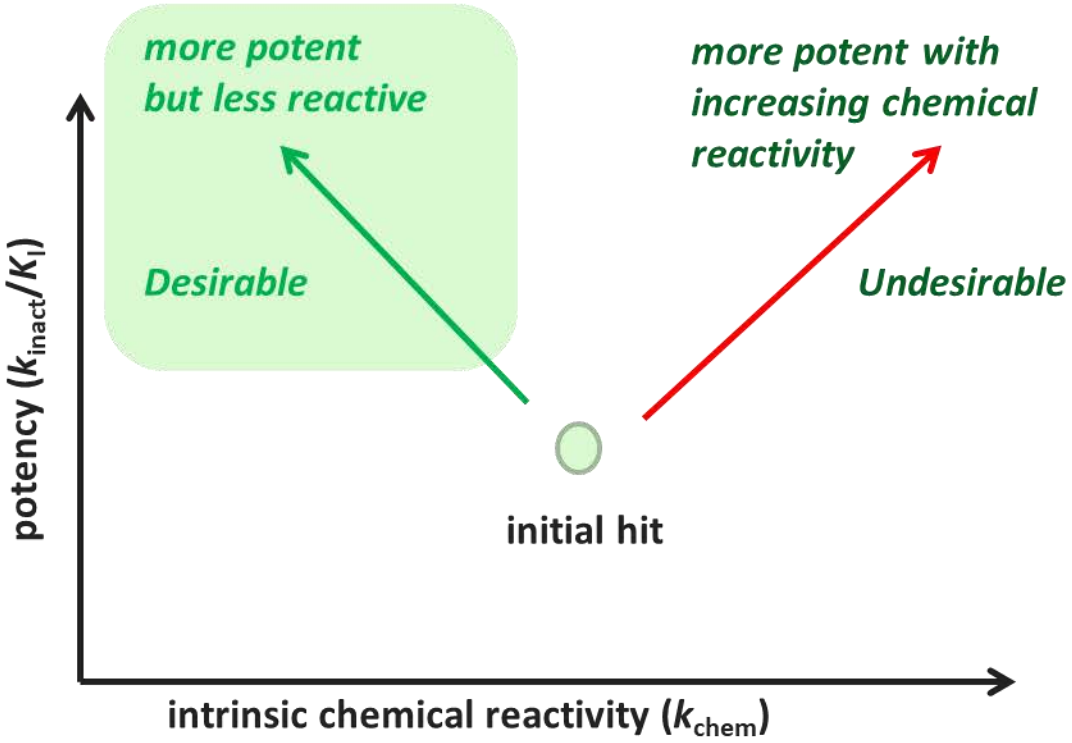
Reaction kinetic analysis of covalent inhibitor-2

簡易的一般反応性試験（蛍光プローブ法による反応性試験）



potency
R; target protein,

intrinsic chemical reactivity
R; GSH



SLAS Discov. 2017 Apr 1:2472555217704654. doi: 10.1177/2472555217704654.
High-Throughput Quantitative Intrinsic Thiol Reactivity Evaluation Using a Fluorescence-Based Competitive Endpoint Assay.

- **一般反応性 K_{chem}が低く、標的への親和性 K_{inact}/Ki の高い化合物　が好ましい。**