Tunable Methacrylamides as New Electrophiles for Covalent Inhibitors

Literature Seminar

M2 Junhao Fu

Outline

- Introduction of covalent inhibitor
- Classification and feature of covalent inhibitor
- Main topic

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Article

Tunable Methacrylamides for Covalent Ligand Directed Release Chemistry

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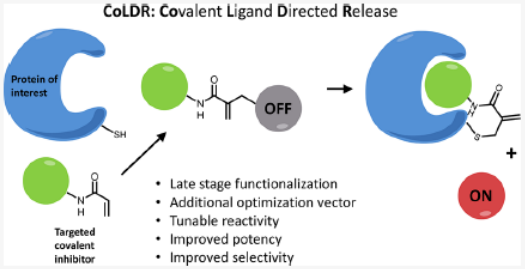
ABSTRACT: Targeted covalent inhibitors are an important class of drugs and chemical probes. However, relatively few electrophiles meet the criteria for successful covalent inhibitor design. Here we describe α-substituted methacrylamides as a new class of electrophiles suitable for targeted covalent inhibitors. While typically α-substitutions inactivate acrylamides, we show that hetero α-substituted methacrylamides have higher thiol reactivity and undergo a conjugated addition−elimination reaction ultimately releasing the substituent. Their reactivity toward thiols is tunable and correlates with the pK_a/pK_b of the leaving group. In the context of the BTK inhibitor ibrutinib, these electrophiles showed lower intrinsic thiol reactivity than the unsubstituted ibrutinib acrylamide. This translated to comparable potency in protein labeling, in vitro kinase assays, and functional cellular assays, with improved selectivity. The conjugate addition−elimination reaction upon covalent binding to their target cysteine allows functionalizing α-substituted methacrylamides as turn-on probes. To demonstrate this, we prepared covalent ligand directed release (CoLDR) turn-on fluorescent probes for BTK, EGFR, and K-Ras^{G12C}. We further demonstrate a BTK CoLDR chemiluminescent probe that enabled a high-throughput screen for BTK inhibitors. Altogether we show that α-substituted methacrylamides represent a new and versatile addition to the toolbox of targeted covalent inhibitor design.



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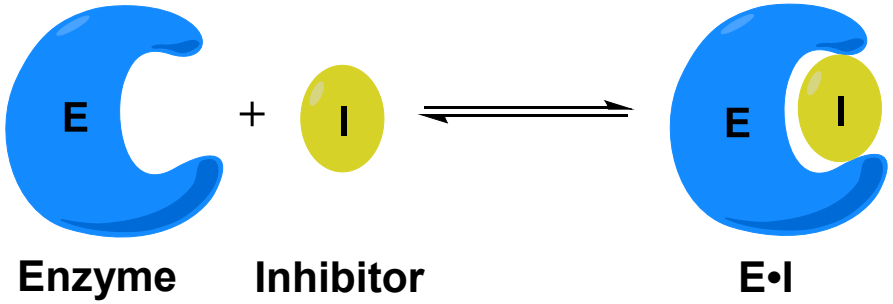
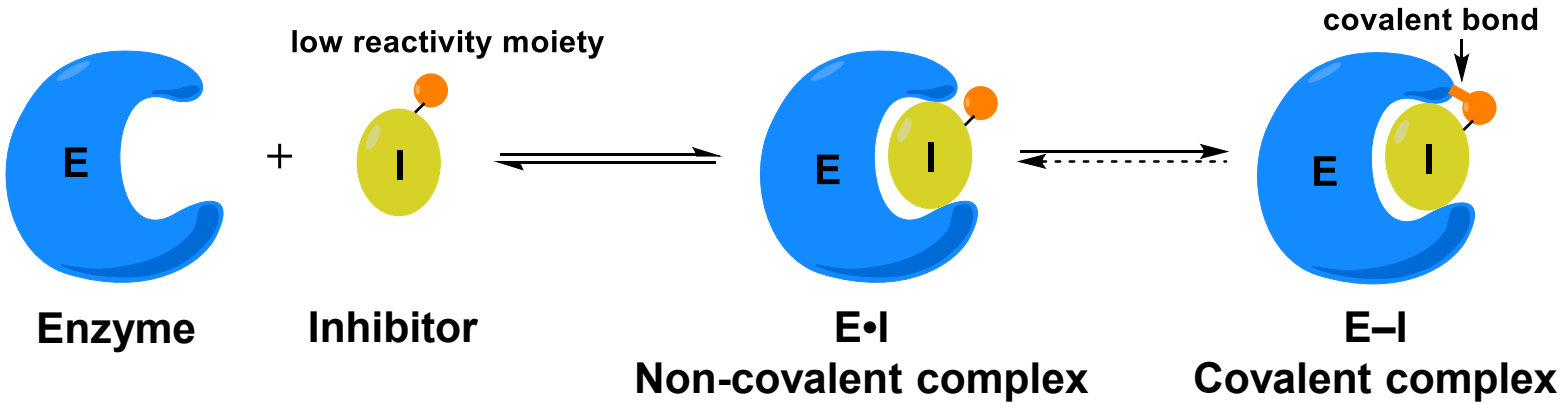
Non-Covalent and Covalent Inhibitors

a) Non-covalent inhibitor



Non-covalent inhibitors bind to their targets in equilibrium and in a reversible manner.

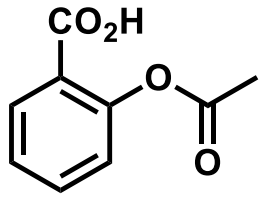
b) Covalent inhibitor



Covalent inhibitors bind to their targets in a two-step manner – the formation of initial non-covalent complex being reversible and formation of final covalent complex being irreversible.

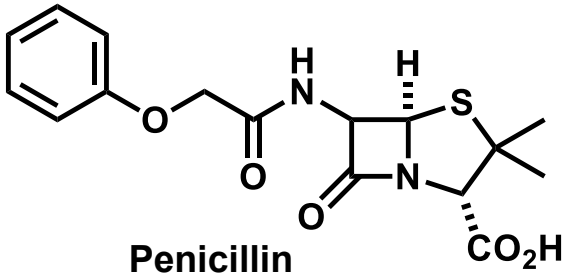
1) Ruddraraju, K. V.; Zhang, Z. *Mol. BioSyst.* **2017**, *13*, 1257.

Timeline of Covalent Inhibitor Drugs



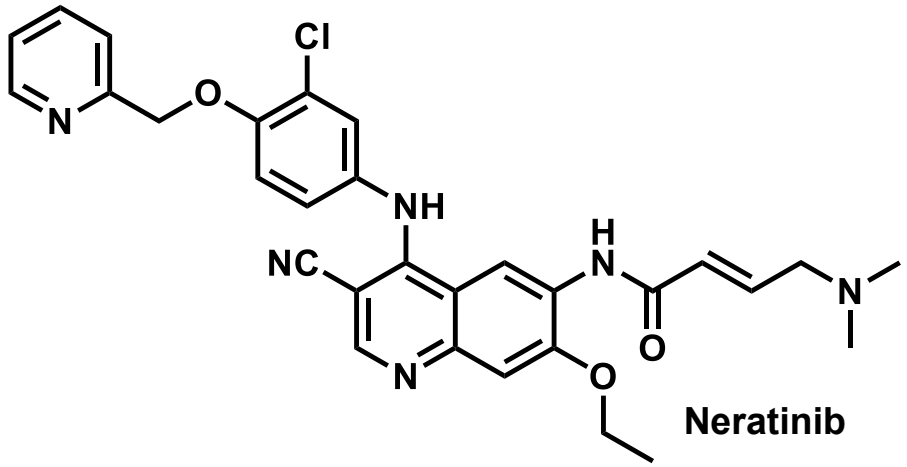
Aspirin

Synthesized by Bayer, inhibitor of cyclooxygenase



Penicillin

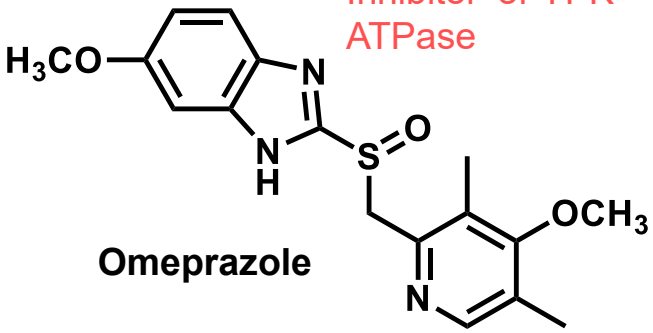
Inhibitor of DD-transpeptidase



Neratinib

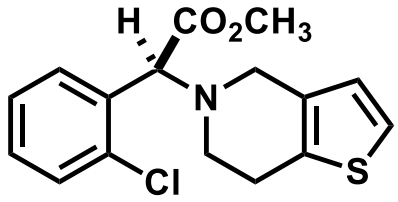
Inhibitor of EGFR

1899 — 1940s — 1980s — 1990s — 2010



Omeprazole

Inhibitor of H^+K^+ ATPase

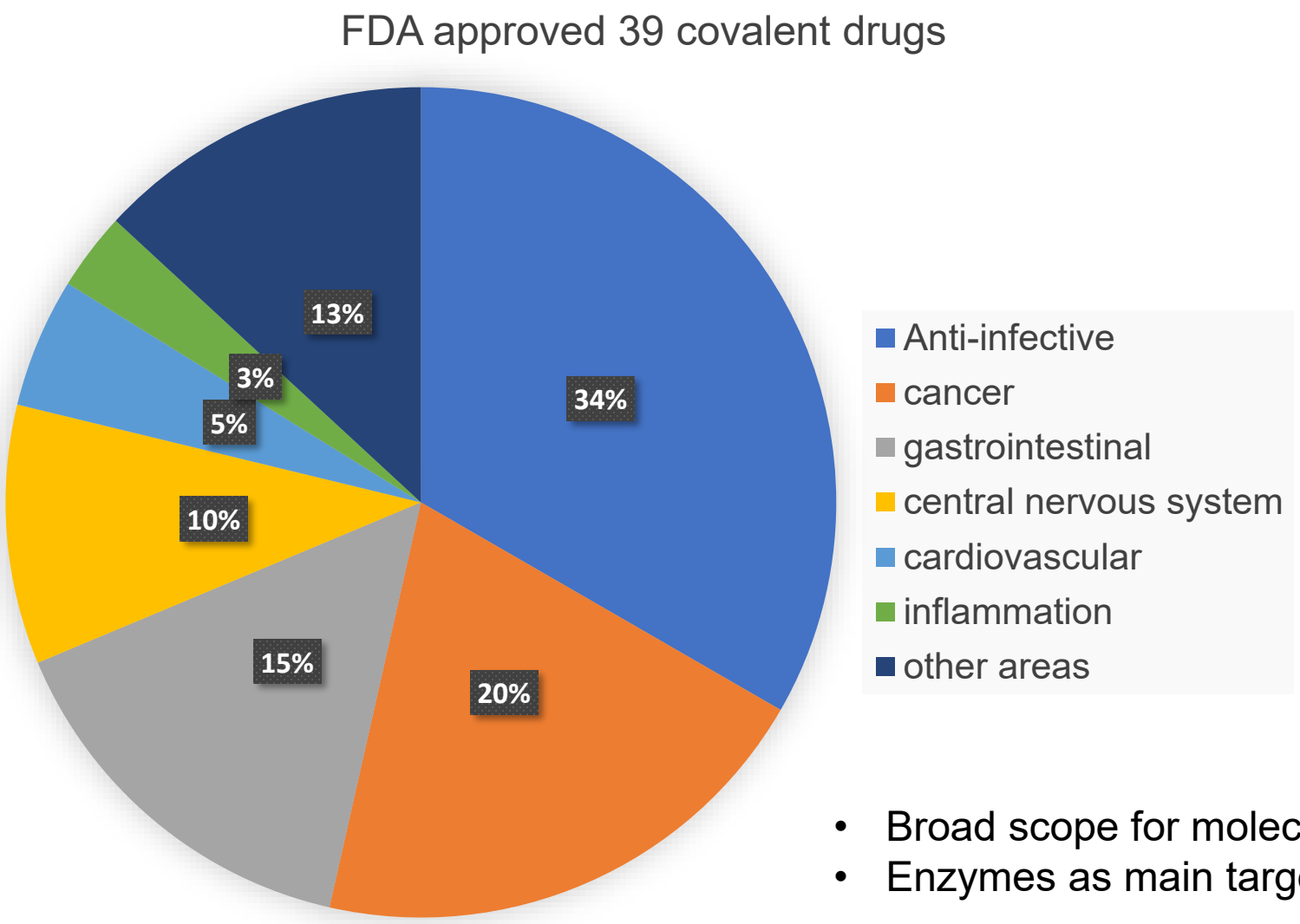


Clopidogrel

$P2Y_{12}$ receptor inhibitor

1) Singh, J.; Petter, R.; Baillie, T.; Whitty, A. *Nat. Rev. Drug Discov.* **2011**, *10*, 307.

Application of Covalent Inhibitor Drugs



- Broad scope for molecular targets
- Enzymes as main targets

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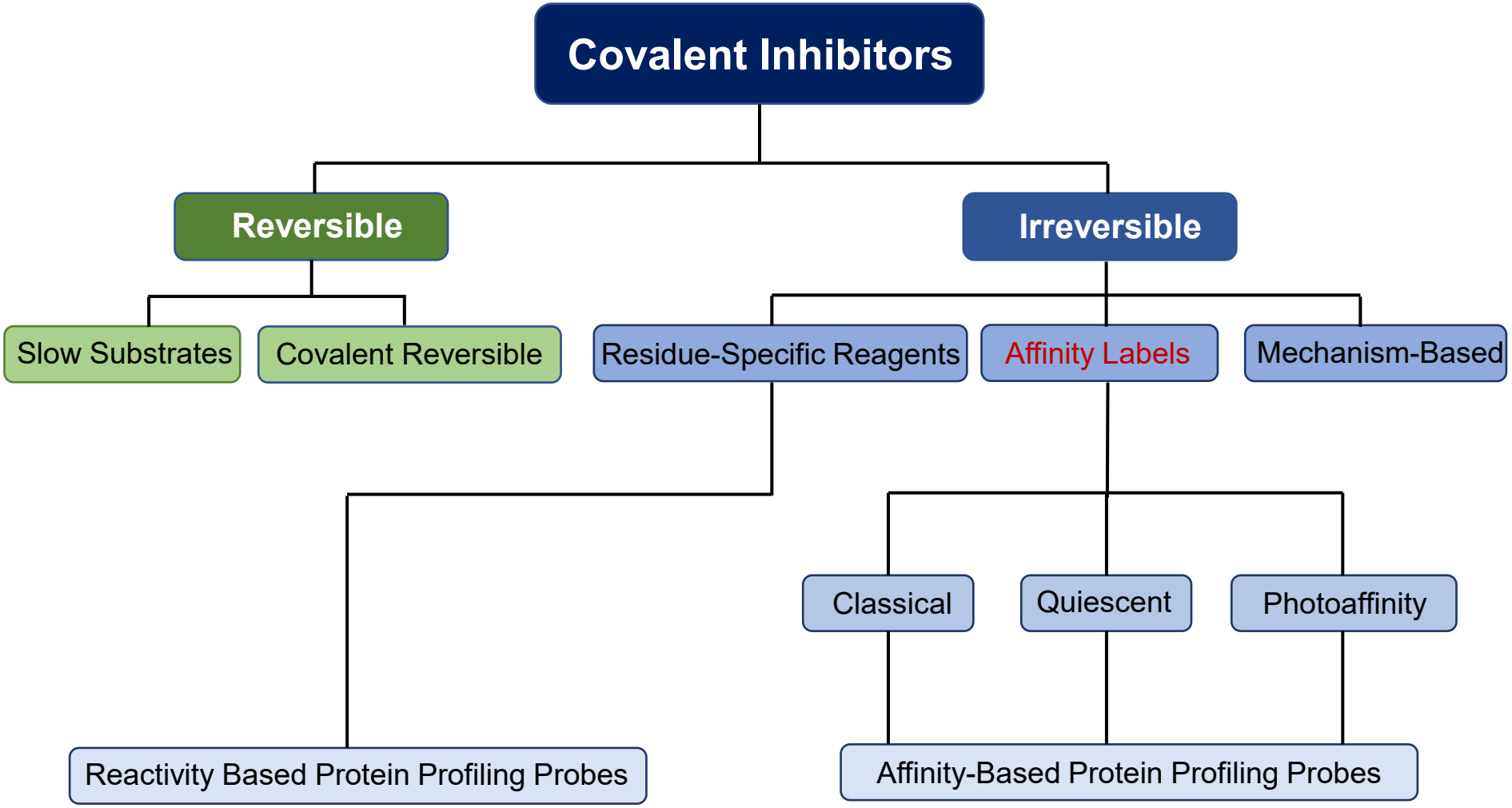
- **Classification and feature of covalent inhibitor**
- Main topic

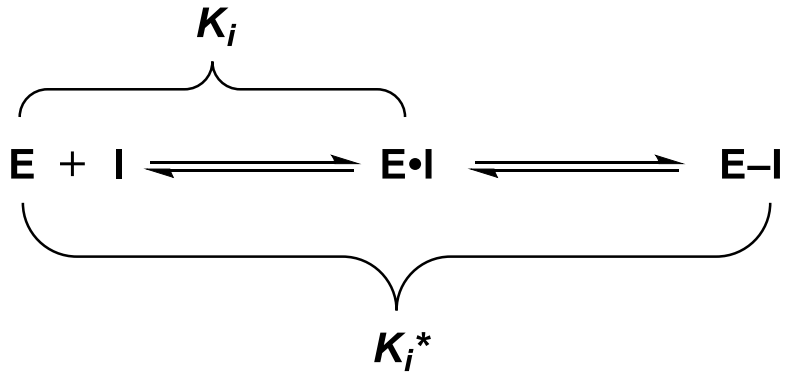
Classification of Covalent Inhibitors

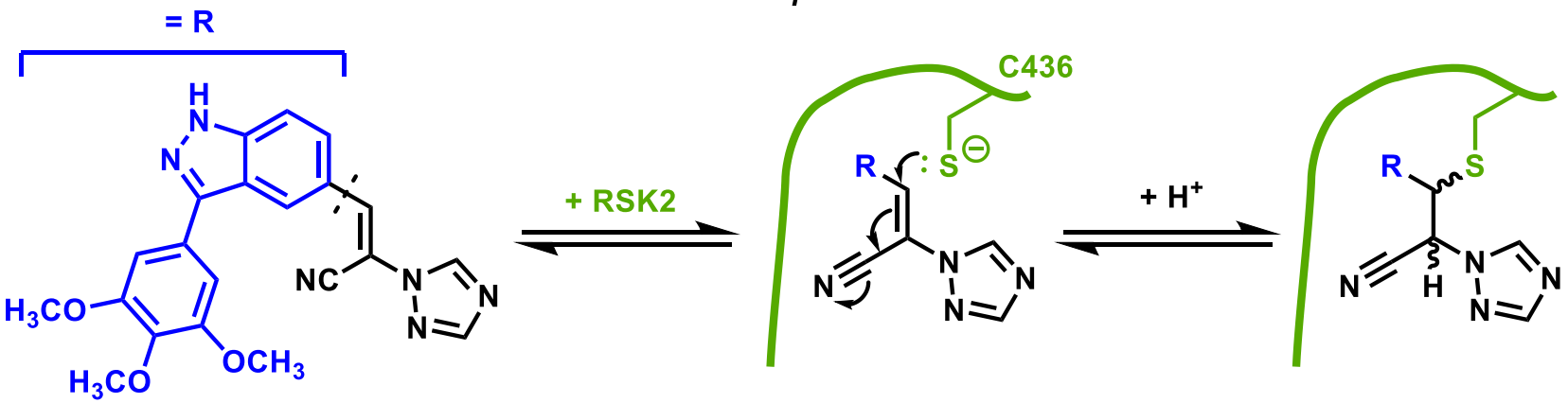


1) Tuley, A.; Fast, W. *Biochemistry.* **2018**, *57*, 3326.

Covalent Reversible Inhibitors

- **Reversible**
- Selective
- K_i* describes the overall dissociation constant of the two steps



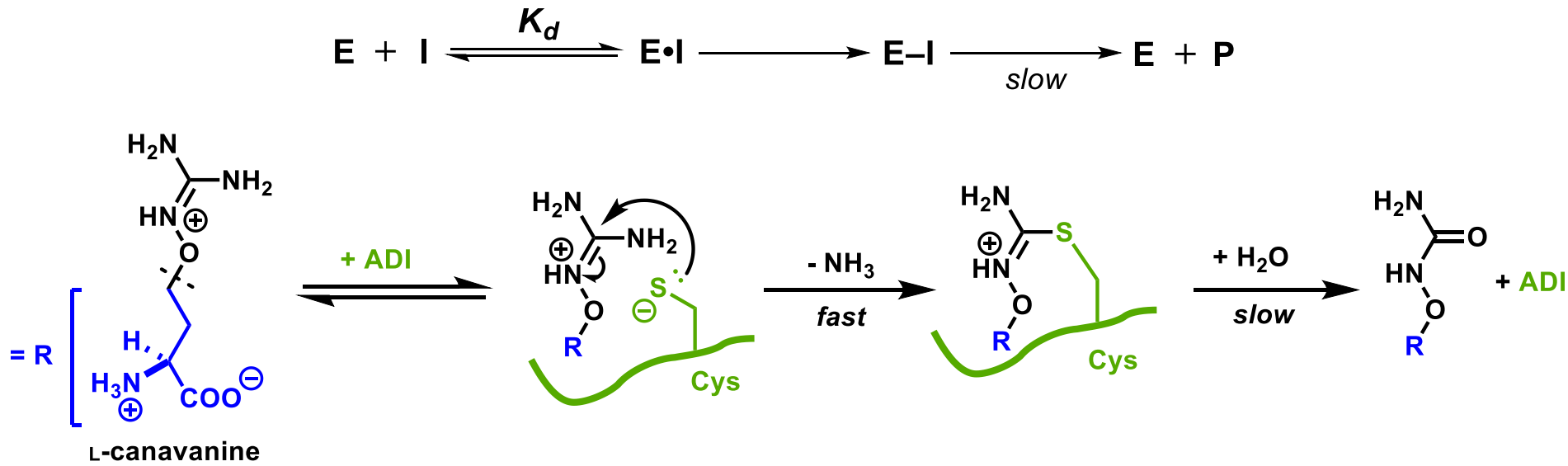


RSK2 = ribosomal protein S6 kinase 2
C436 = 436th Cys residue (non-catalytic)

The low pK_a of the α-proton makes the reaction reversible.

Slow substrates

- **Reversible**
- Inhibitor recognized as substrate for the enzyme
- Covalent intermediate further decomposes into free enzyme and non-active product (P)



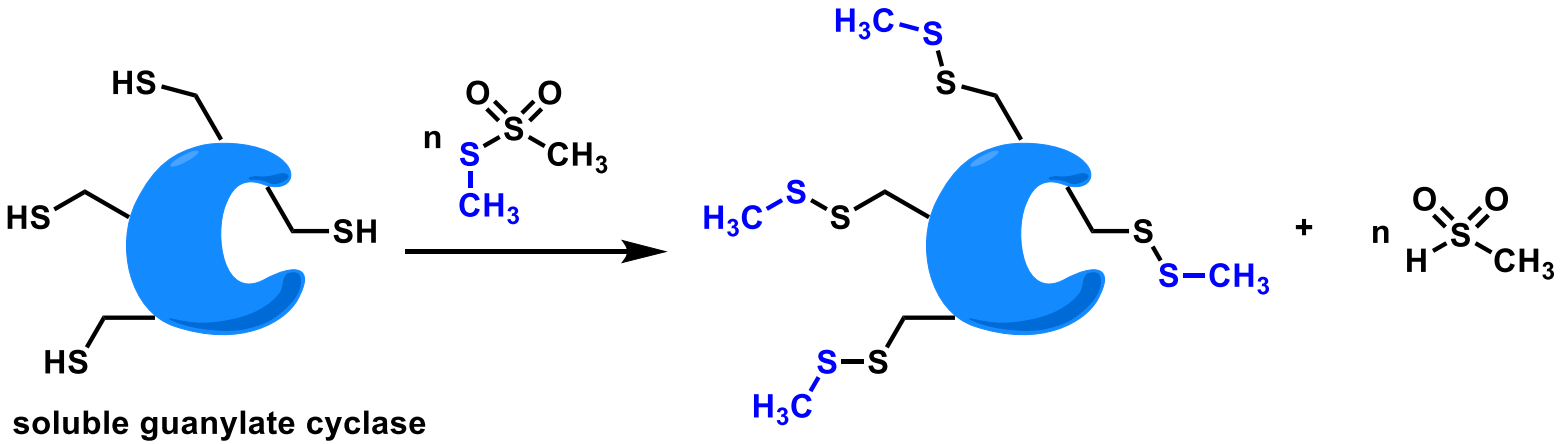
ADI = *Pseudomonas aeruginosa* arginine deiminase

Slow hydrolysis of the pseudo thiourea through the normal catalytic mechanism leads to release of O-ureido and recovered active enzyme.

Residue-Specific Reagents

- **Irreversible**
- The least selective
- Used only in vitro as biochemical tools
- Influenced by chemo selectivity for particular nucleophiles instead of non-covalent affinity

$$E + I \longrightarrow E\text{–}I$$

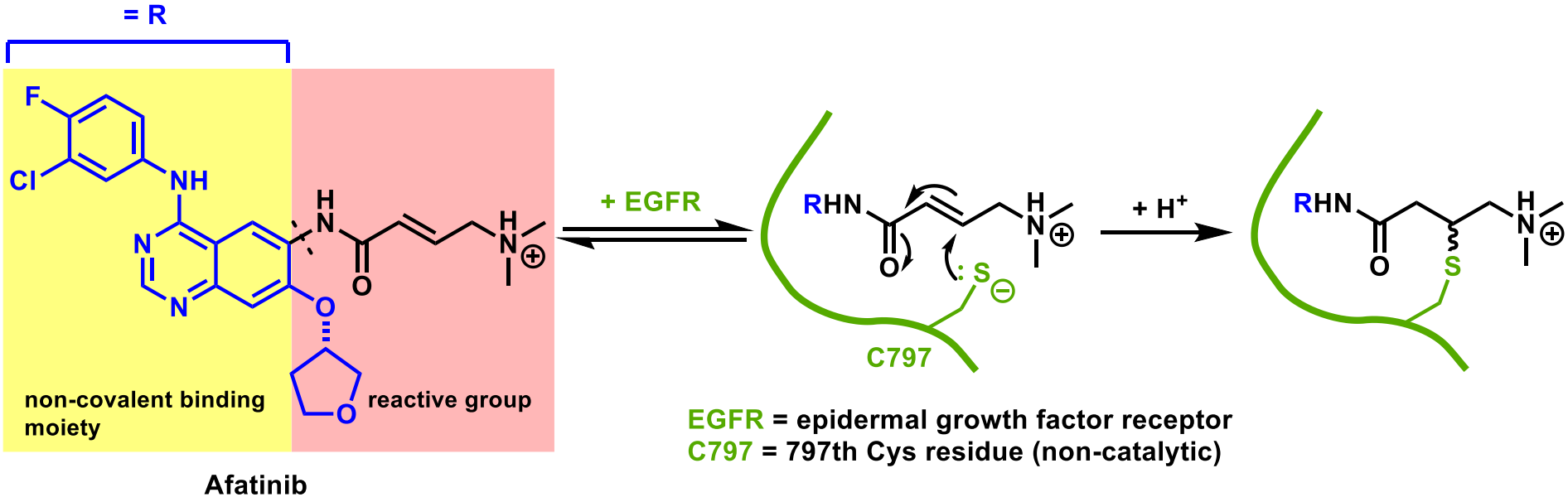


High concentration leads to nonspecific enzyme inhibition, illustrating the nonselective nature of residue-specific reagents.

Affinity Labels

- **Irreversible**
- Site selective inhibition
- Moiety with non-covalent binding affinity + reactive group (typically a poor electrophile)
- Dissociation from covalent complex E–I to non-covalent complex E•I can be ignored

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E{\bullet}I \xrightarrow{k_{inact}} E\text{–}I$$

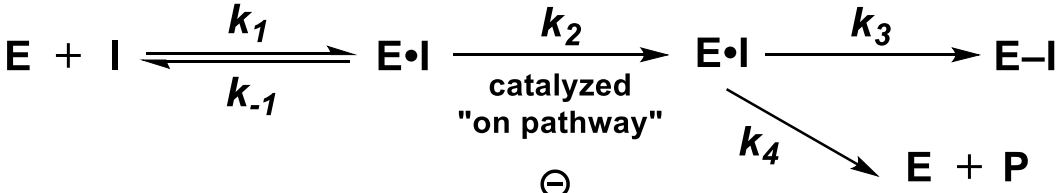


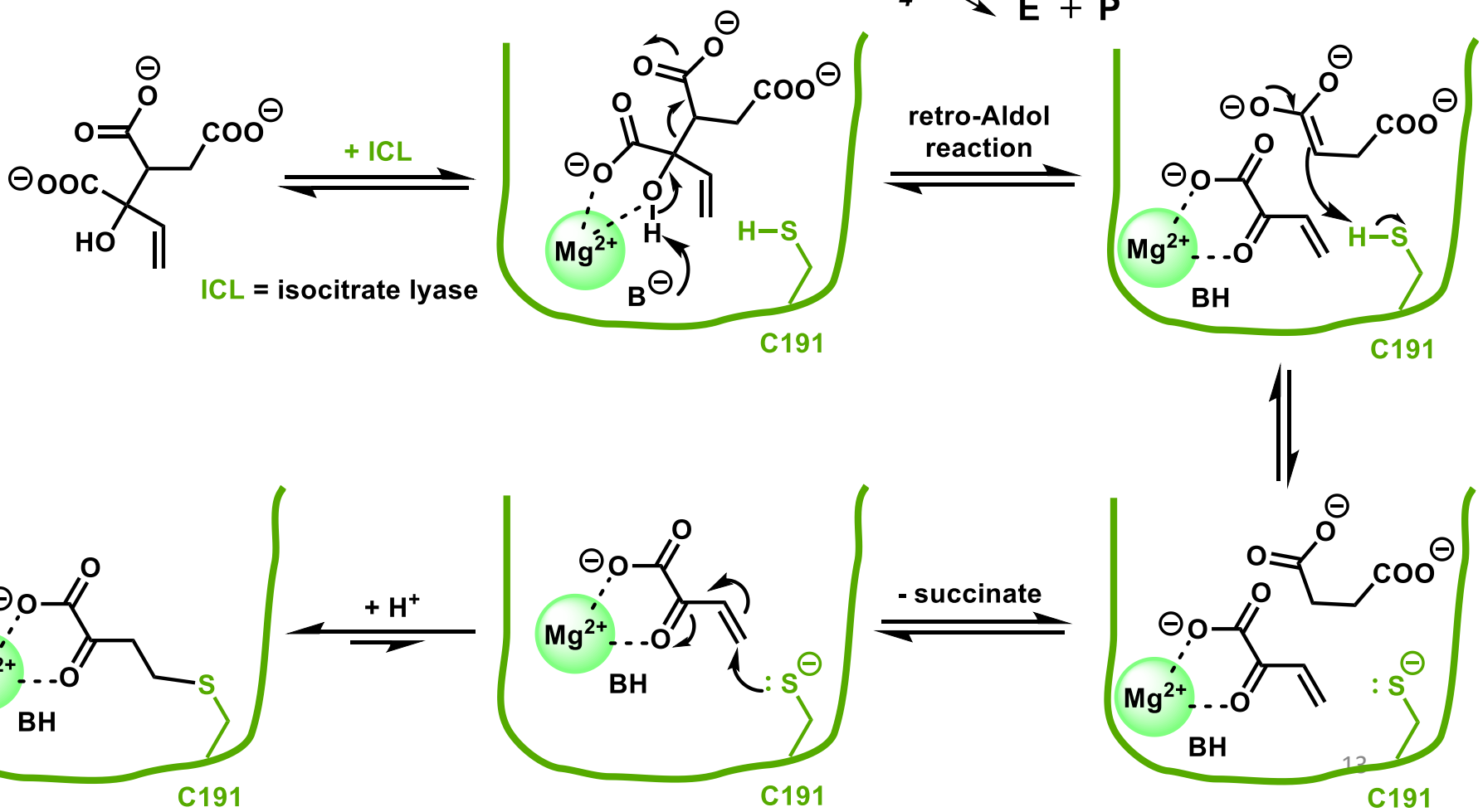
EGFR = epidermal growth factor receptor
C797 = 797th Cys residue (non-catalytic)

The effective molarity of the reactive group near the site of enzyme modification is raised by the non-covalent binding.

Mechanism-Based Enzyme Inactivators

- **Irreversible**
- Selectively Bind to active site of enzymes
- Processed by catalytic mechanism to give reactive species





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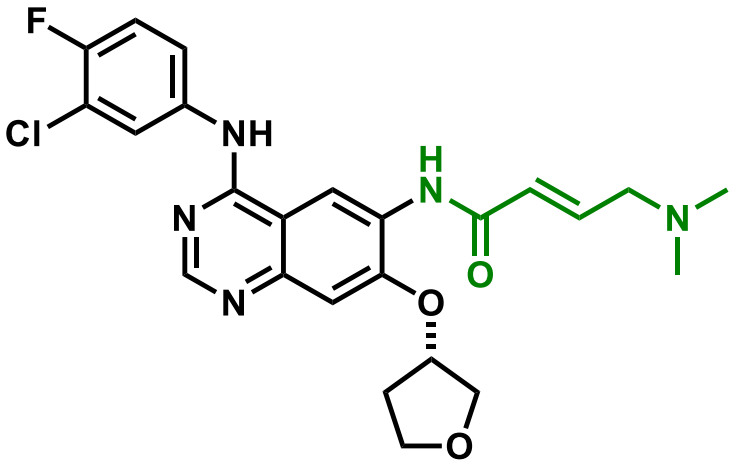
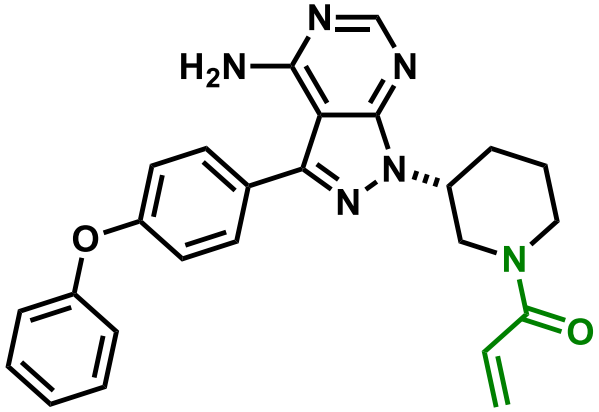
Acrylamide-Based Covalent Inhibitors



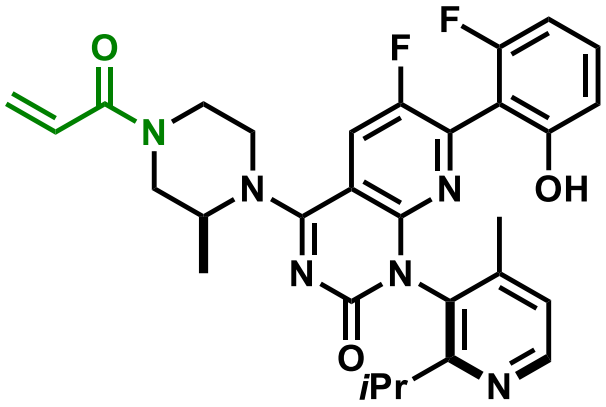
Afatinib

Inhibitor of EGFR



Ibrutinib

Inhibitor of BTK (Bruton's tyrosine kinase)



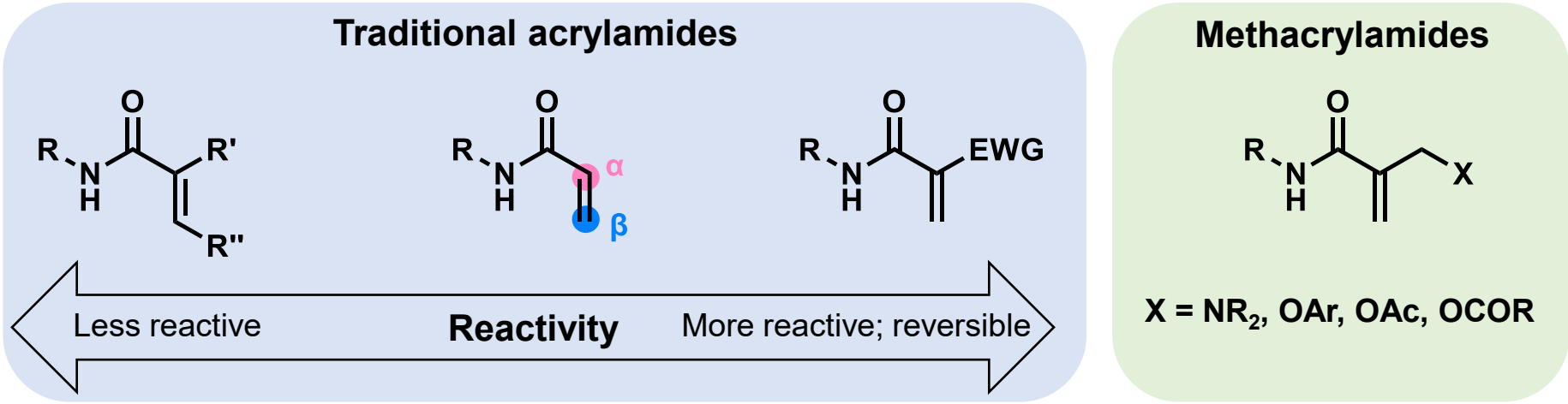
AMG-510

Inhibitor of K-Ras

- Used as electrophiles
- Nonequilibrium kinetics
- Full target occupancy
- Flexibility to modify the structure for ADME (absorption, distribution, metabolism and excretion)
- Tunability by structure modification

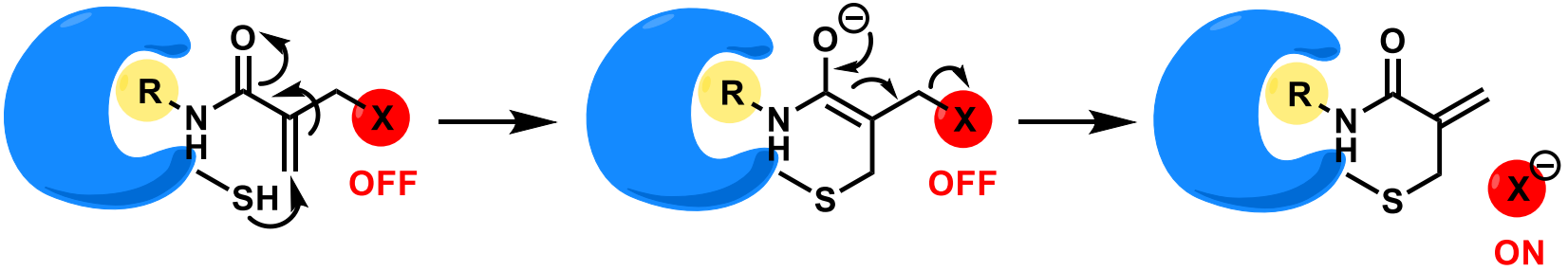
Tunable Methacrylamides

Structure modification of acrylamides:



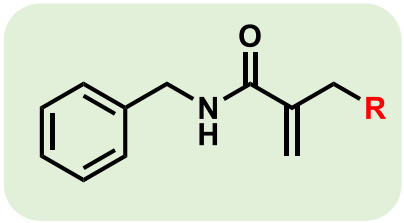
Various acrylamide substitutions can modify its intrinsic reactivity and reversibility.

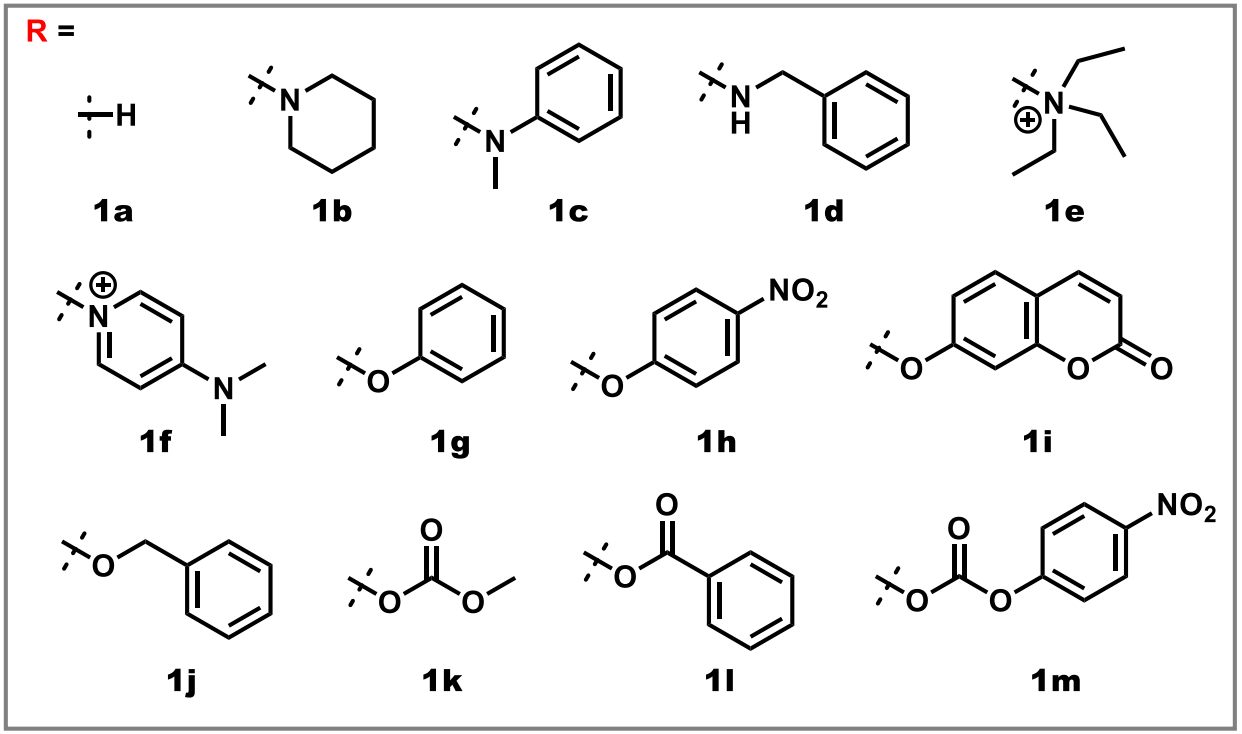
Schematic representation for covalent ligand directed release chemistry:



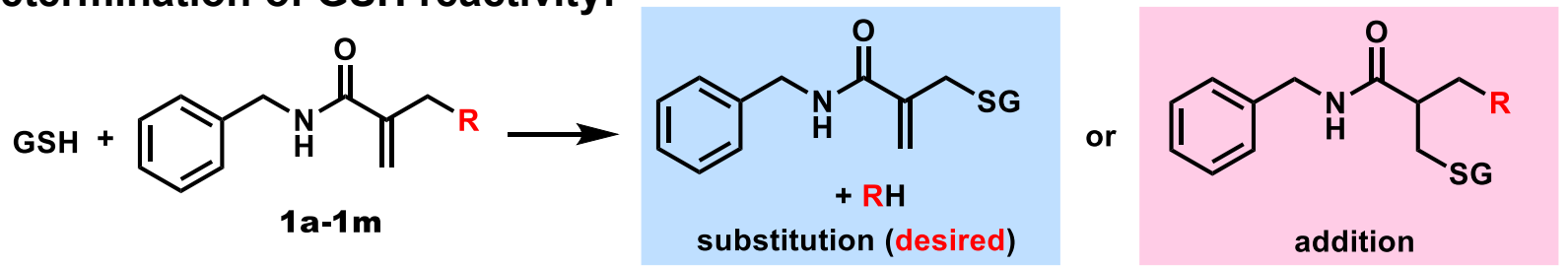
Moreover, targeted covalent inhibitors are also modified into turn-on fluorogenic, chemiluminescent or other functionalized probes.

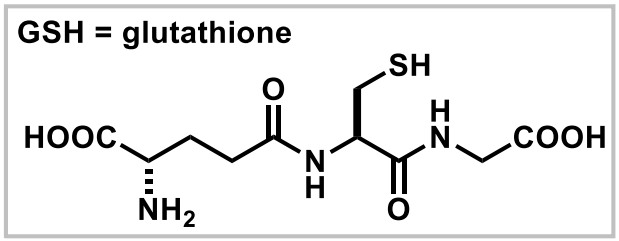
Model α-substituted Methacrylamides





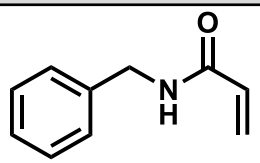
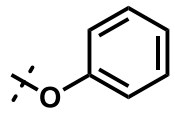
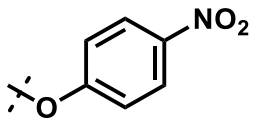
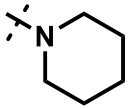
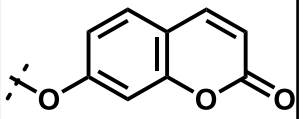
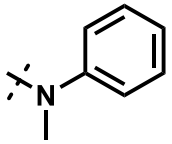
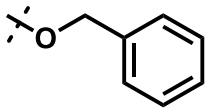
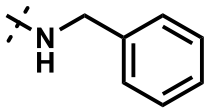
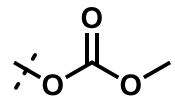
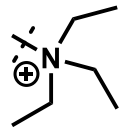
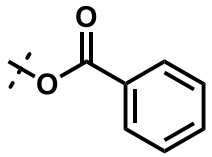
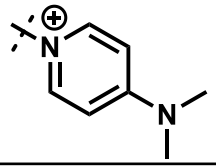
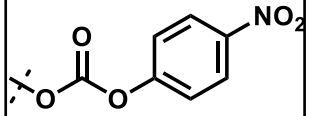
Determination of GSH reactivity:





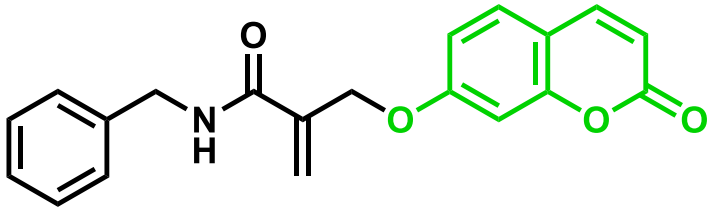
Depletion of the starting material was quantified by LC/MS to determine the $t_{1/2}$ of model compounds to GSH.

Model α-substituted Methacrylamides

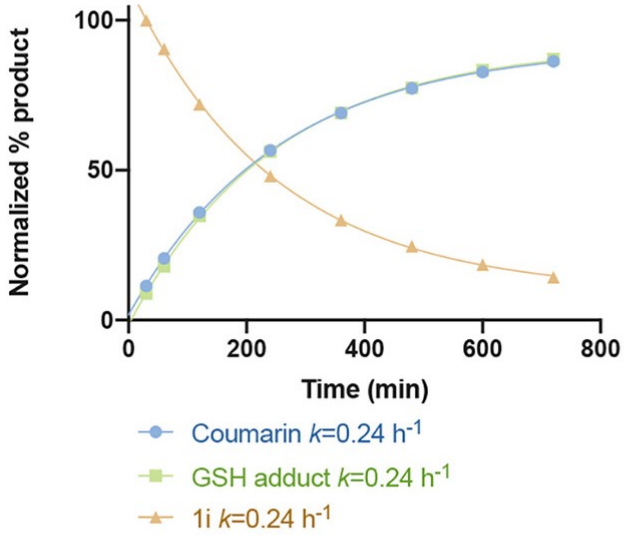
Compound	R =	$t_{1/2}$ to GSH (h)	Substitution /Addition	Compound	R =	$t_{1/2}$ to GSH (h)	Substitution /Addition
[BnA]		>100	Addition	**1g**		9.9	Substitution
1a	**H**	>100	Neither	**1h**		2.6	Substitution
1b		0.3	Mixed	**1i**		3.9	Substitution
1c		66	Substitution	**1j**		>100	Addition
1d		0.7	Mixed	**1k**		1.1	Substitution
1e		0.1	Substitution	**1l**		1.6	Substitution
1f		5.0	Mixed	**1m**		N/A[a]	Substitution

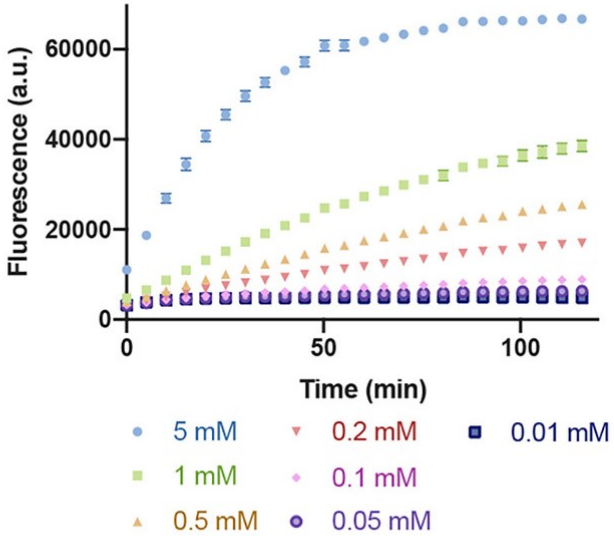
[a]The compound reacts through a two-step mechanism.

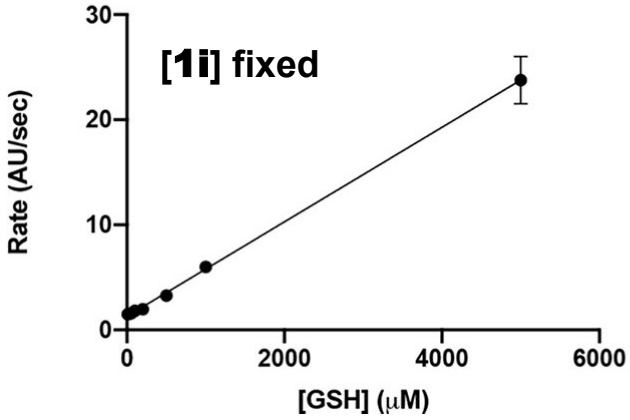
7-hydroxy coumarin as turn-on fluorescent probe

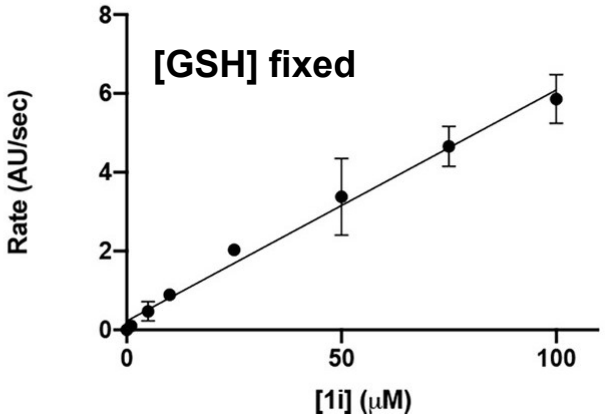


1i





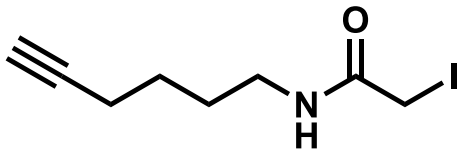




- The rates of coumarin formation, GSH adduct formation and depletion of **1i** were consistent.
- By following the coumarin fluorescence, the reaction rate can be monitored.
- Linearity between reaction rate and concentration of **1i** and GSH was observed.

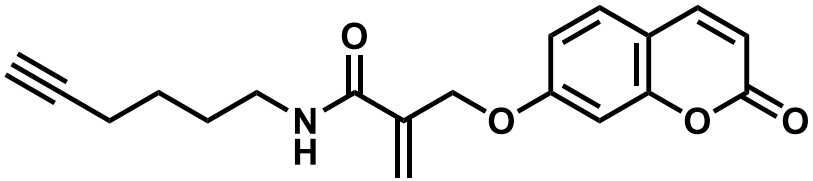
Proteomic Reactivity

Model electrophilic alkyne probes:

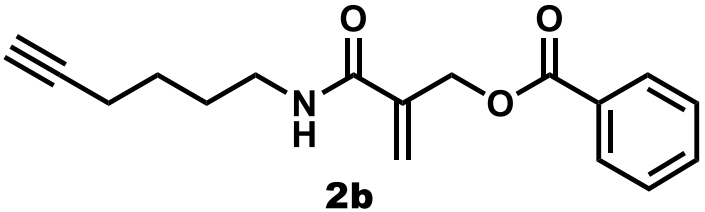


positive control

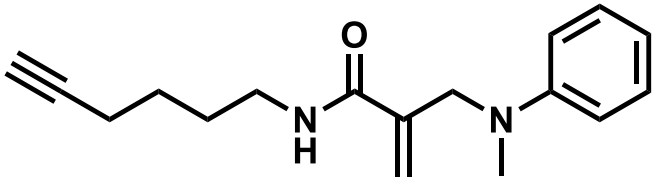
IA-alkyne



2a



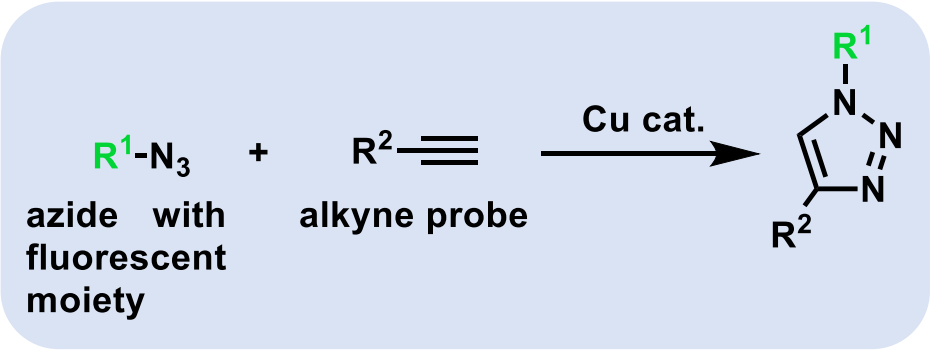
2b



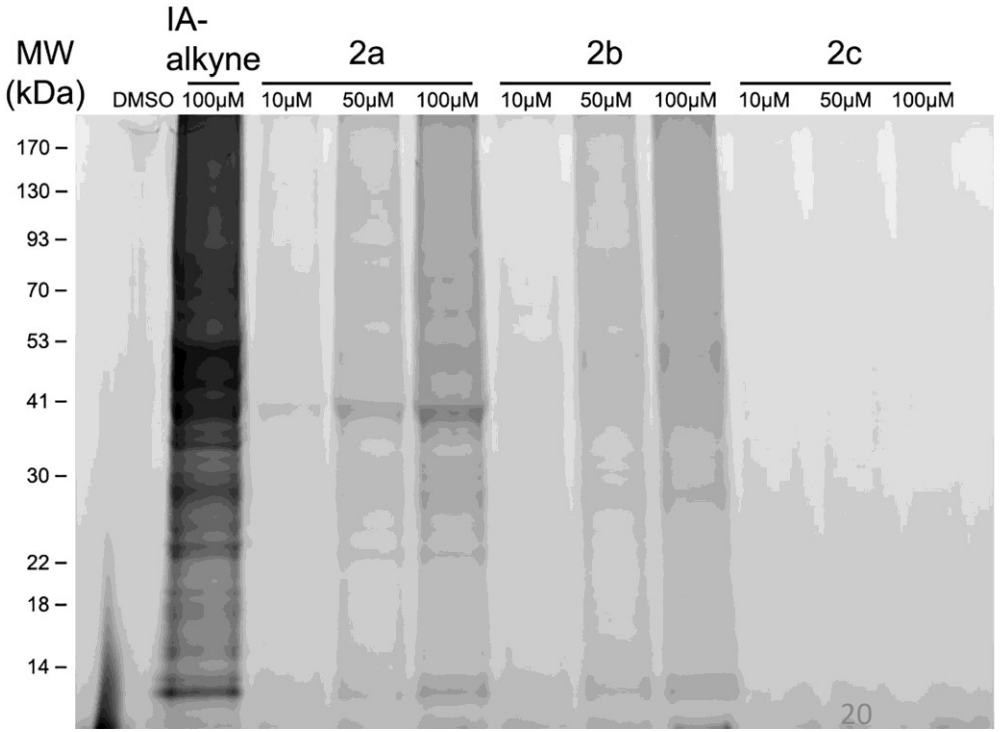
2c

- Molecular recognition of coumarin of **2a** to proteins might raise the ability for labeling.
- Consistently, **2c** showed low activity similar to the result of GSH experiment.

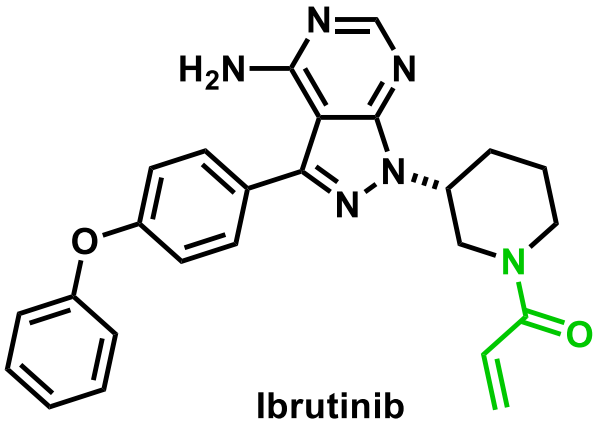
Cu-catalyzed 'click chemistry' for labeling :

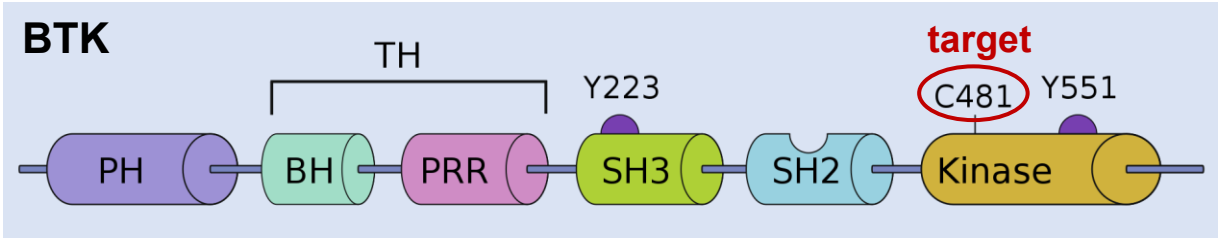


In situ proteomic labeling:

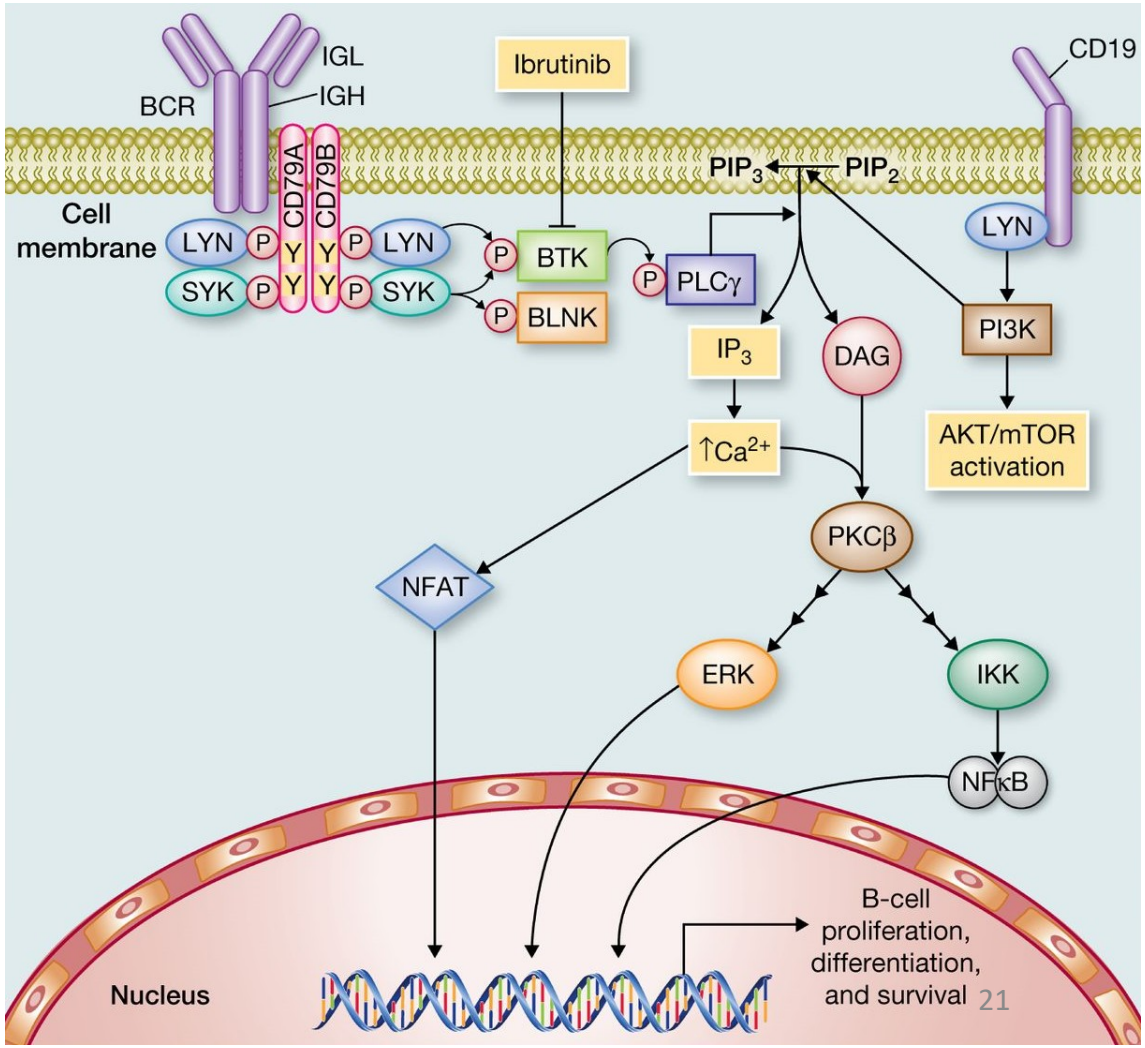


Ibrutinib – Covalent Kinase Inhibitor of BTK



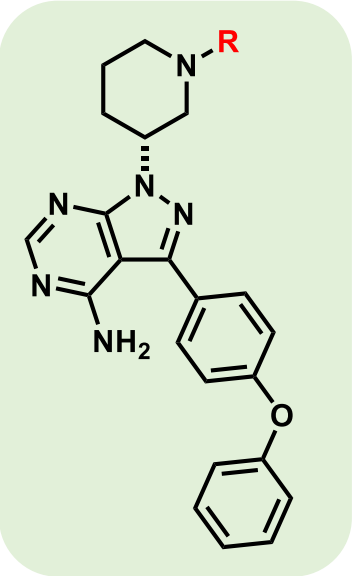


- **FDA approved targeted covalent inhibitor drug**
- **Inhibitor of BTK (Bruton's tyrosine kinase)**
- **Disrupts BCR downstream signaling, leading to cell apoptosis in B cell malignancy cell lines**
- **Treatment for B cell cancers (mantle cell lymphoma, chronic lymphocytic leukemia, etc.)**

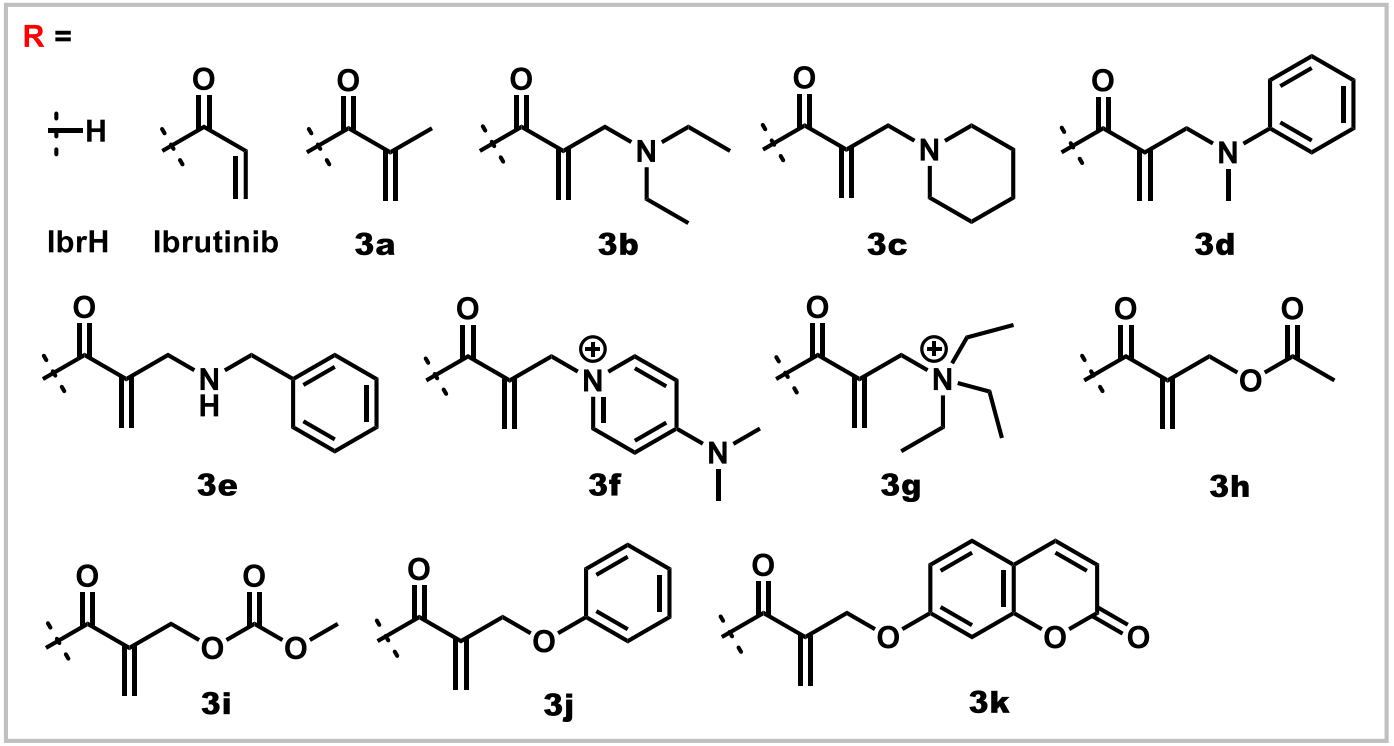


1) Herrera, A.; Jacobsen, E. *Clin. Cancer Res.* **2014**, *20*, 5365.

Activity Evaluation of Ibrutinib-Based Inhibitors

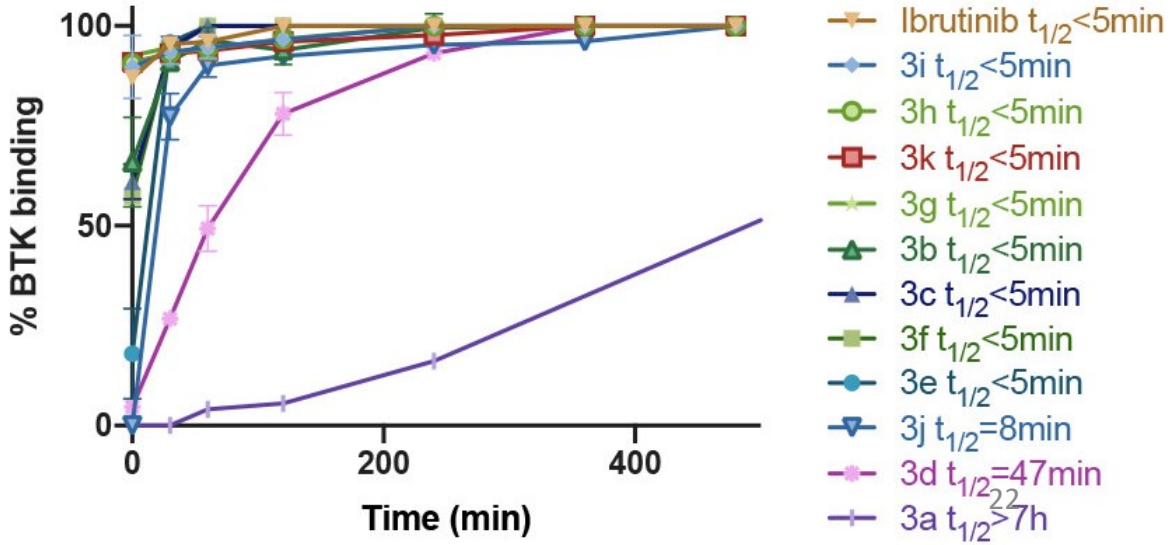


R =



Time course LC-MS binding to BTK assay:

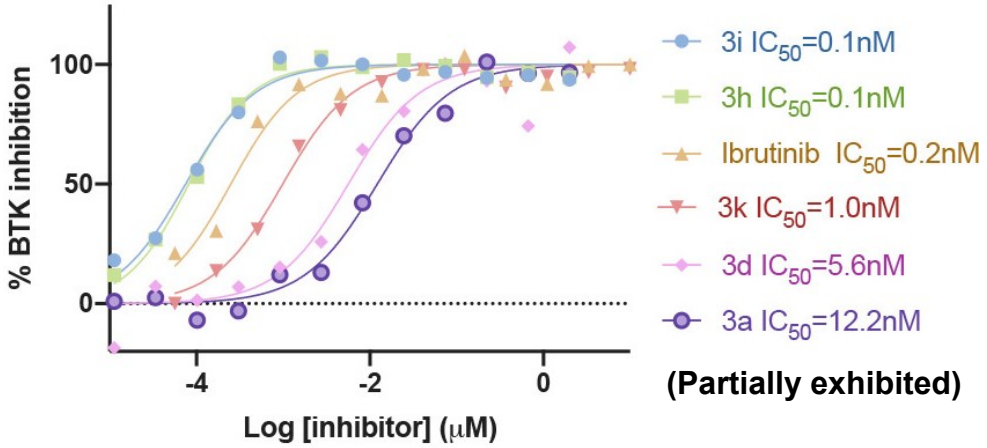
- Most compounds showed comparable activity to ibrutinib.
- **3j** and **3d** labeled BTK the slowest, consistent with the results of model compounds.



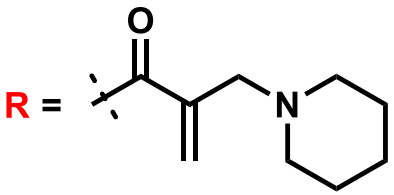
Activity Evaluation of Ibrutinib-Based Inhibitors

In vitro kinase activity assay:

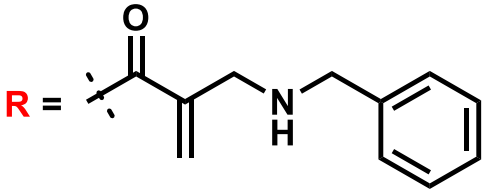
- **3c**, **3h**, **3i** and **3e** showed smaller IC_{50} values than ibrutinib (IC_{50} = 288 pM)
- Most other compounds inhibited BTK with IC_{50} < 1nM



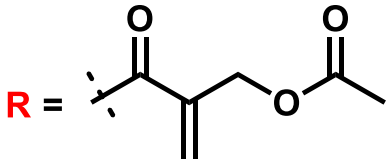
(Partially exhibited)



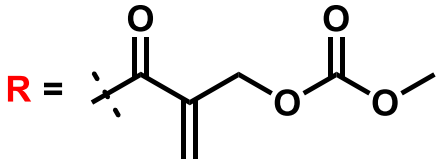
3c



3e



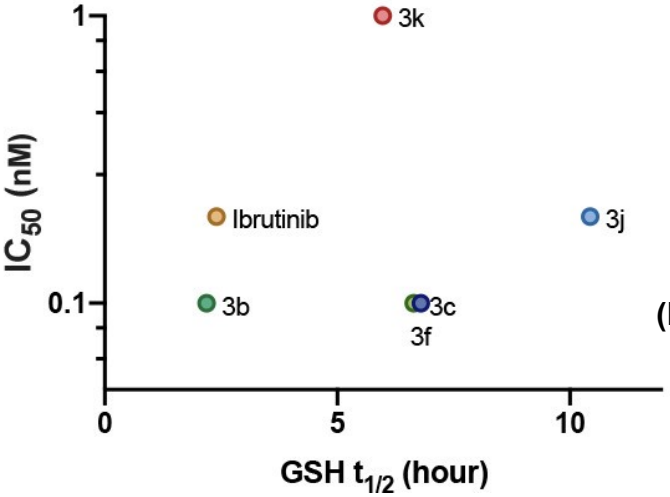
3h



3i

GSH-based reactivity assay:

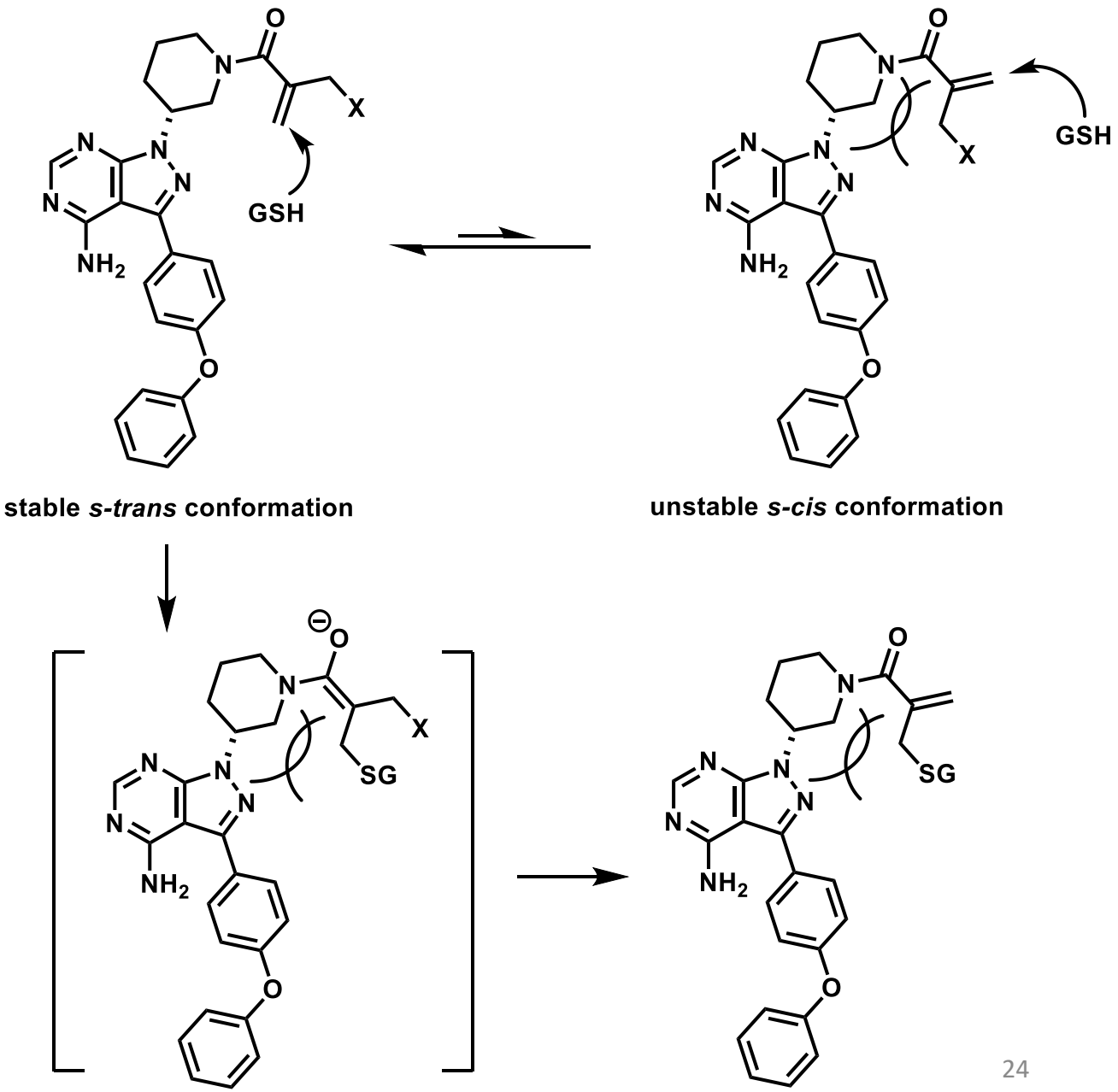
- Despite being potent in previous assays, these compounds showed lower reactivity than ibrutinib
- Possibly due to steric hindrance around the Michael acceptor



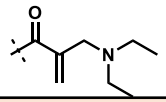
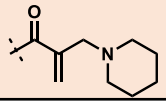
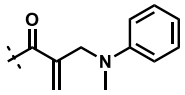
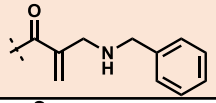
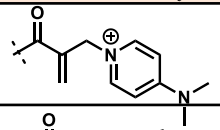
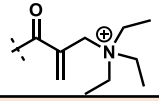
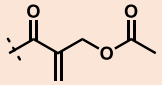
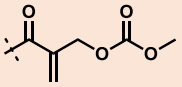
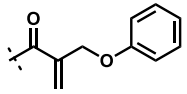
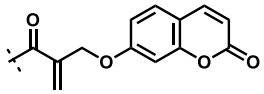
(Partially exhibited)

GSH Reactivity of Ibrutinib-Based Inhibitors

The fixed geometry of acrylamide facilitates faster reaction with BTK for these ibrutinib derivatives while making it difficult for GSH to reach the acrylamide due to steric hindrance.

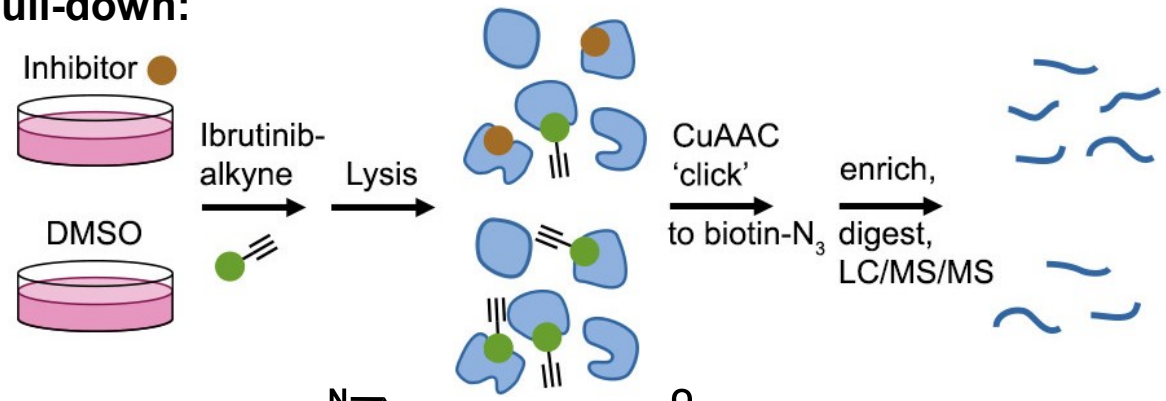


Activity Evaluation of Ibrutinib-Based Inhibitors

compound	R =	BTK $t_{1/2}$ (min)	BTK reaction	BTK IC_{50} (nM)	GSH $t_{1/2}$ (h)	GSH reaction
Ibrutinib		<5	Addition	0.2	2	Addition
3a		>420	Addition	12.2	No reaction	Addition
3b		<5	Mixed	0.1	2	Mixed
3c		<5	Addition	0.1	7	Mixed
3d		47	Substitution	5.6	>100	Substitution
3e		<5	Addition	0.1	>100	Substitution
3f		<5	Mixed	0.1	7	Mixed
3g		<5	Substitution		0.1	Substitution
3h		<5	Substitution	0.1	59	Substitution
3i		<5	Substitution	0.1	12	Substitution
3j		8	Substitution	0.2	10	Substitution
3k		<5	Substitution	1.0	6	Substitution

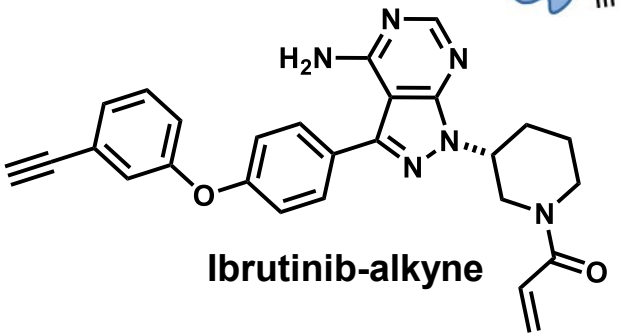
Selectivity of Ibrutinib-Based Inhibitors

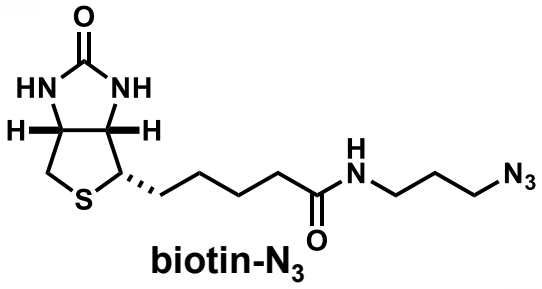
Pull-down:

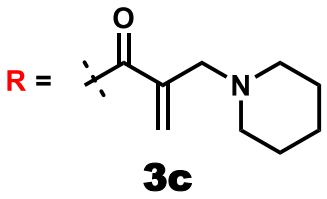


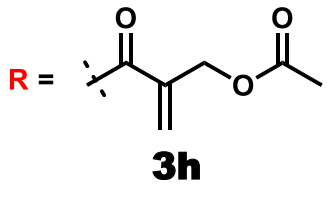
Inhibition of protein by ibrutinib derivatives ends up with lower intensity

Decline in intensity reveals degree of inhibition

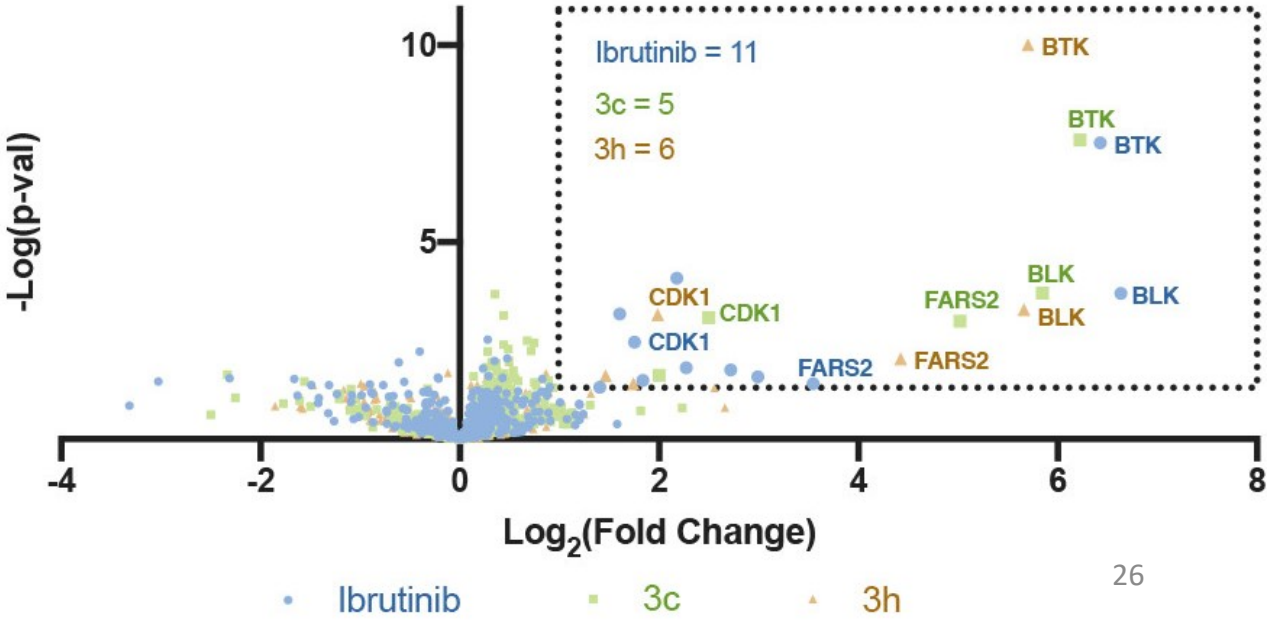




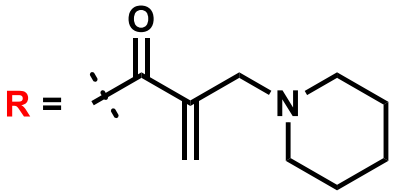




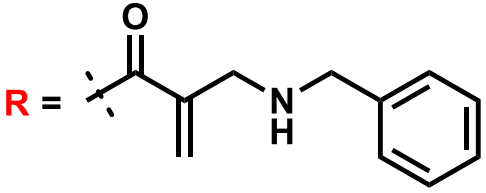
- **BTK as well as known off-targets BLK, TEC, and CDK1 were observed.**
- **Ibrutinib labeled slightly higher number of significant targets than 3c and 3h.**



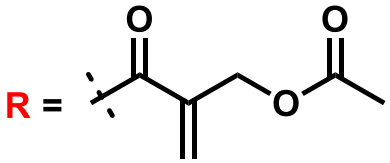
Selectivity of Ibrutinib-Based Inhibitors



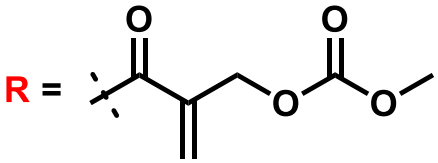
3c



3e



3h



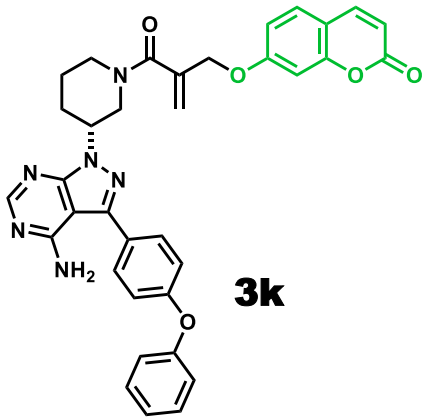
3i

compound	BTK	BLK[a]		BMX[a]		EGFR[a]		ERBB2[a]		ITX[a]	
	IC_{50} (nM)	IC_{50} (nM)	BLK/ BTK	IC_{50} (nM)	BMX/ BTK	IC_{50} (nM)	EGFR /BTK	IC_{50} (nM)	ERBB2 /BTK	IC_{50} (nM)	ITK/ BTK
Ibrutinib	0.3	0.1	0	0.2	1	3	10	10	38	78	311
3c	0.1	0.1	1	0.3	5	7	105	33	472	43	607
3h	0.1	0.6	7	0.3	4	28	348	196	2400	295	3613
3e	0.1	0.1	1	0.4	5	3	36	13	169	30	383
3i	0.1	0.8	11	0.4	5	31	417	172	2292	232	3087

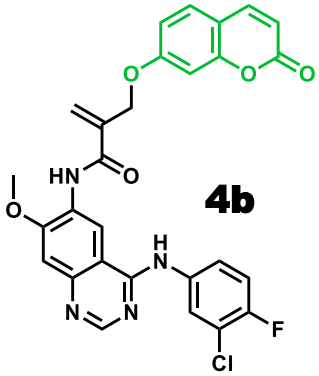
[a] off-targets

- **All the compounds showed improved selectivity to BTK compared with ibrutinib.**
- **3h and 3i showed much higher selectivity over ERBB2 and ITX than ibrutinib.**

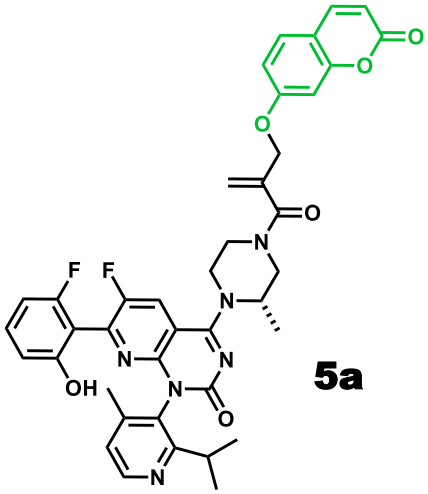
Covalent Ligand Directed Release Chemistry



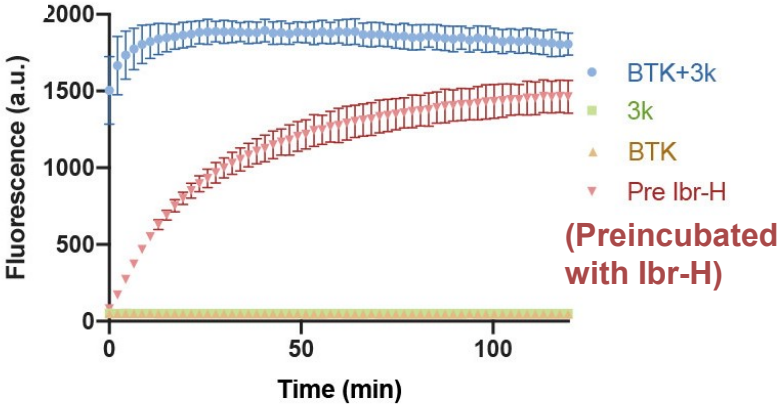
3k

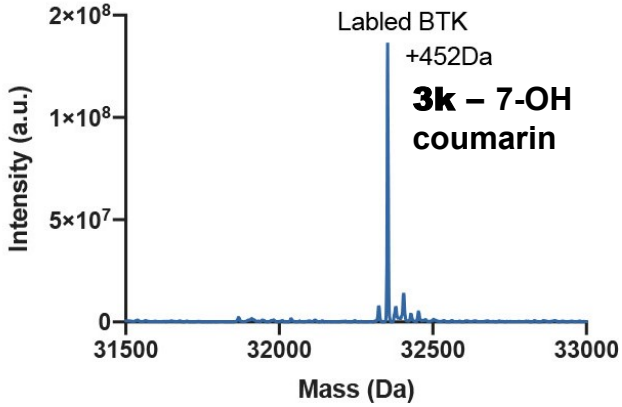


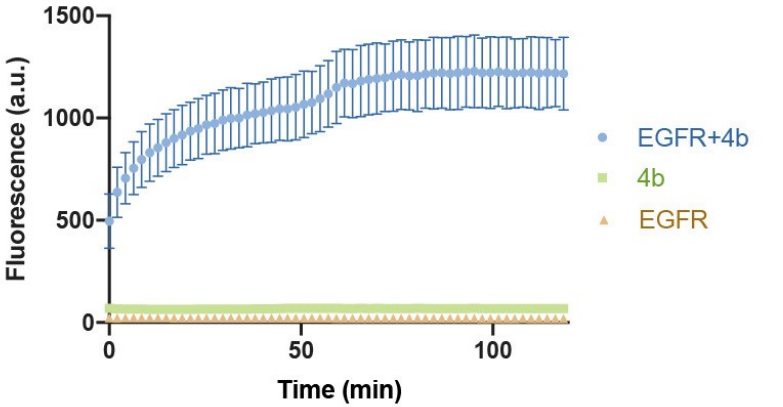
4b

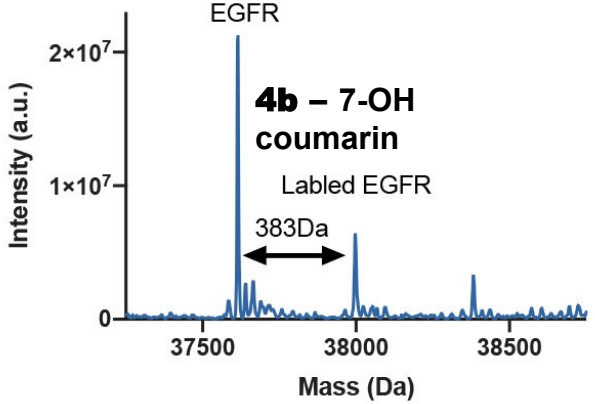


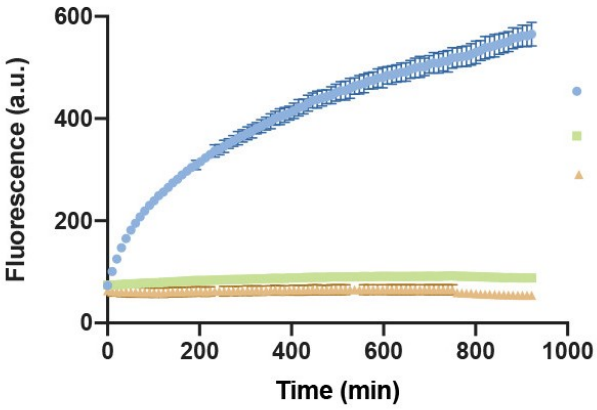
5a

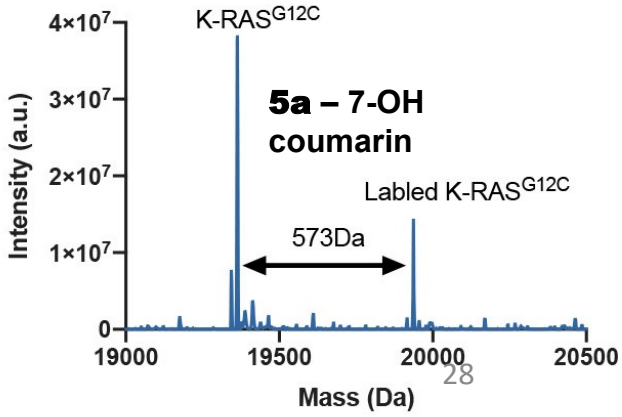






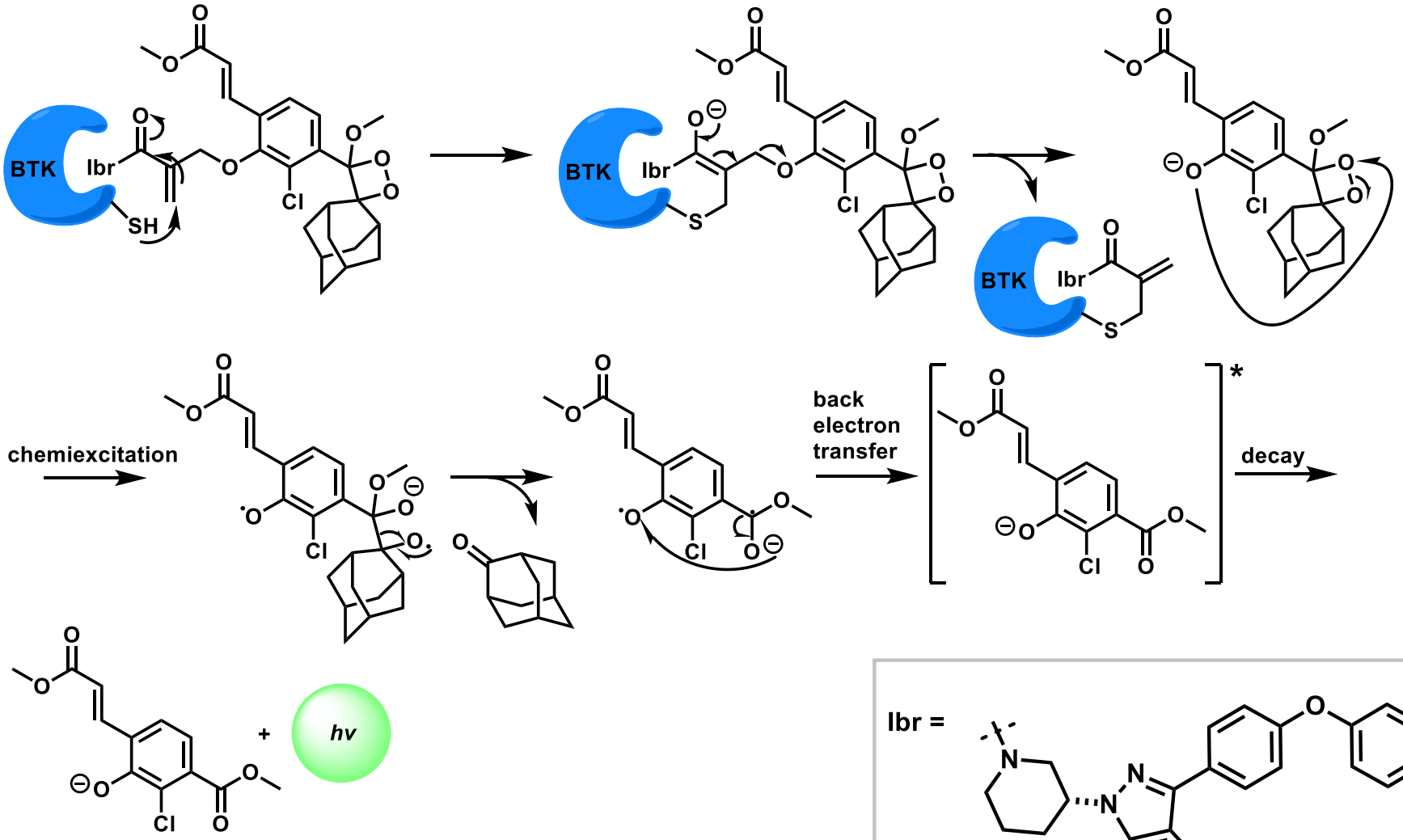






Covalent Ligand Directed Release Chemistry

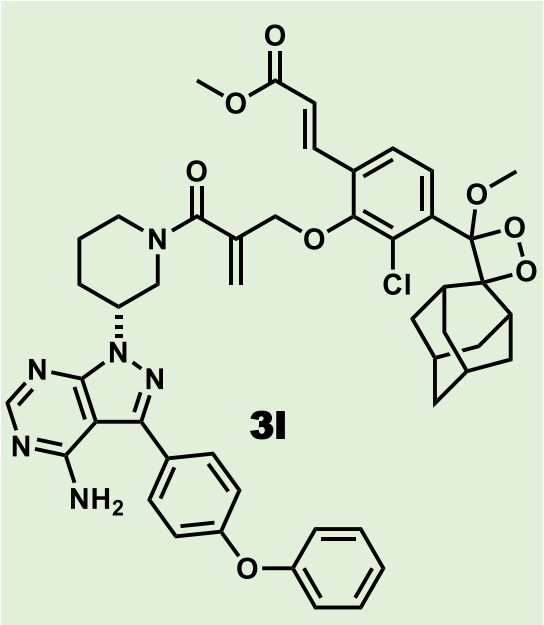
Adamantylidene-dioxetane-based chemiluminescent turn-on probes



Emission of a photon in the chemiexcitation of the phenolate-dioxetane intermediate could be used for sensing and imaging of the enzymes.

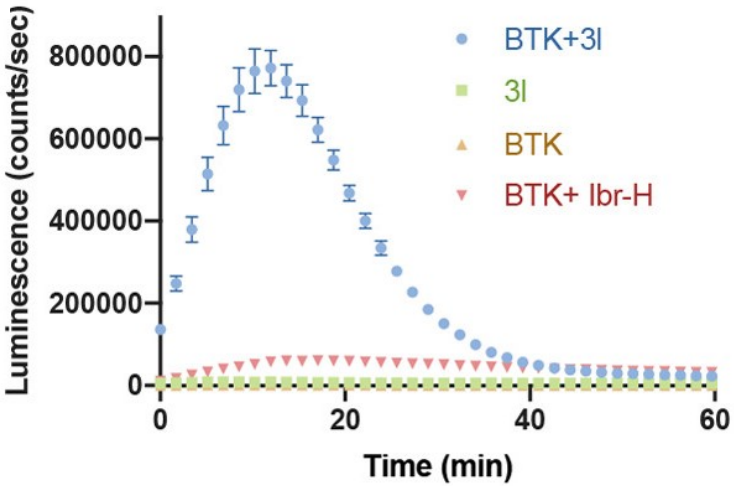
1) Hananya, N.; Boock, E.; Bauer, C.; Fainaro, R.; Shabat, D. *J. Am. Chem. Soc.* **2016**, *138*, 13438.

Covalent Ligand Directed Release Chemistry

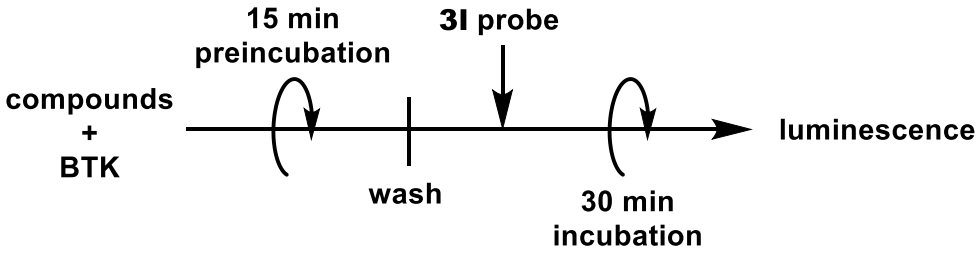


Time dependence of luminescence signal:
Only upon mixing of probe and target is increase in luminescence observed.

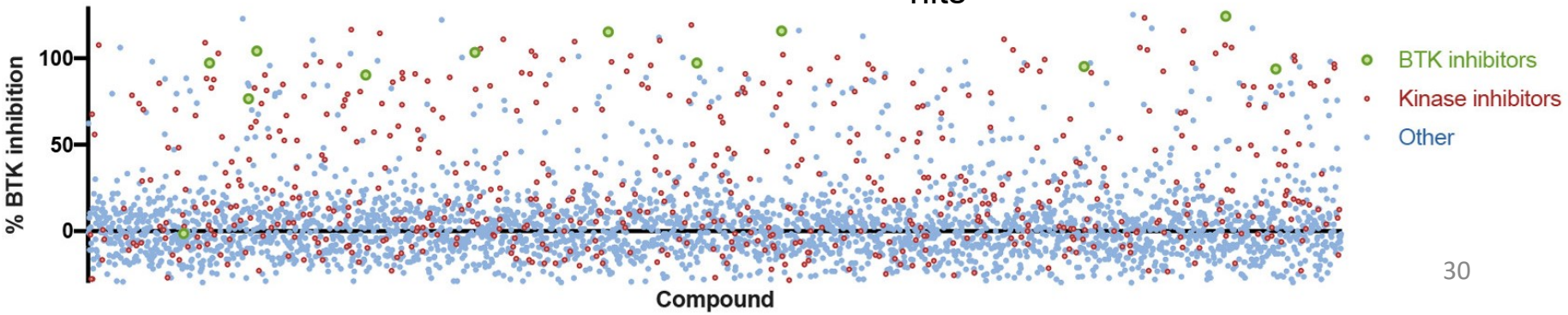
Ability for measurement of BTK binding



High-throughput screen for BTK inhibitors with 3725 bioactive compounds:

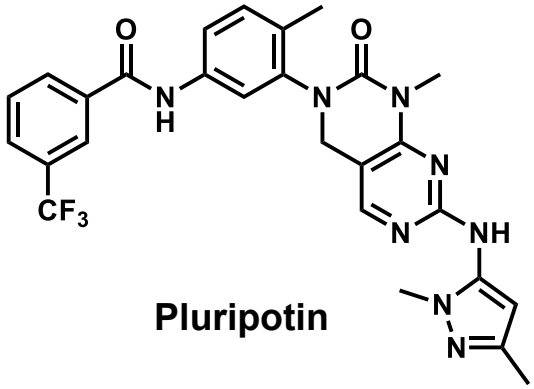


- 216 (6%) strong hits (>70 % inhibition)
- 121/216 strong hits → known kinase inhibitors
- 11/12 BTK inhibitors identified as strong hits

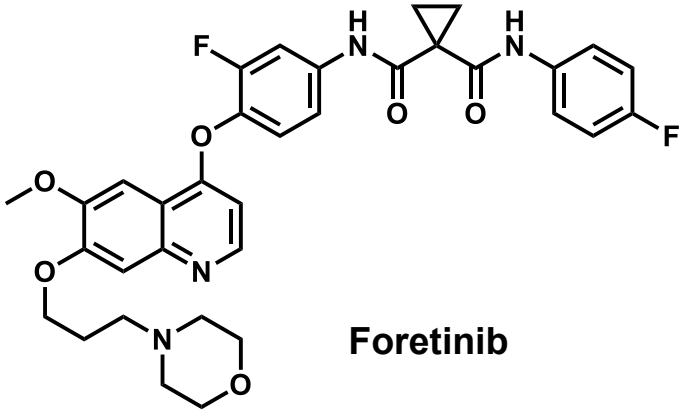


Covalent Ligand Directed Release Chemistry

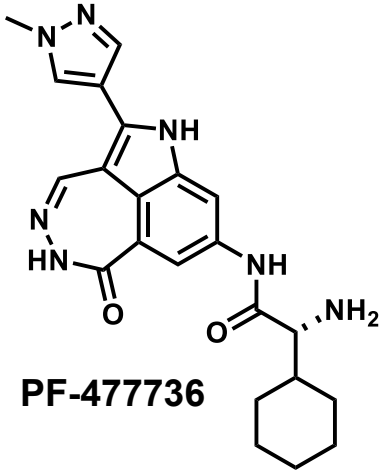
Newly recognized BTK inhibitors



Pluripotin



Foretinib



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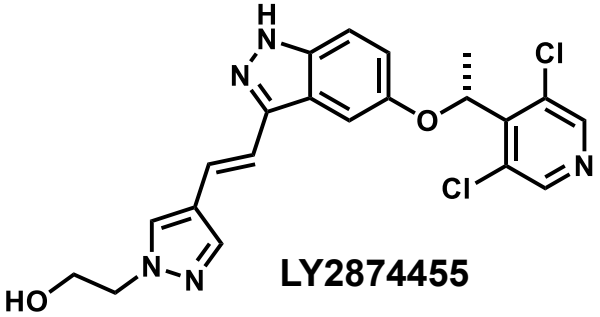
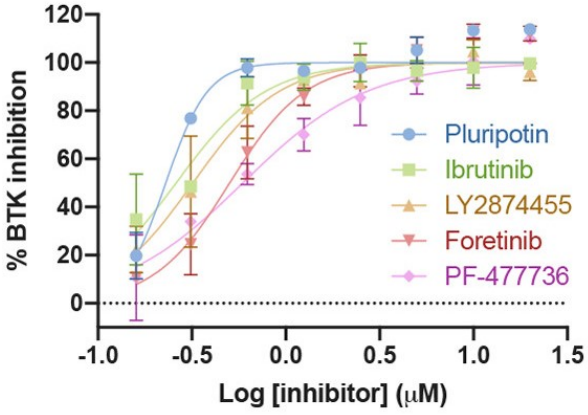
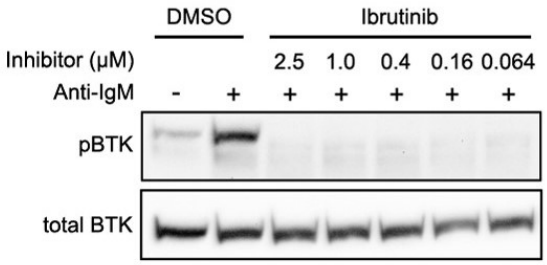
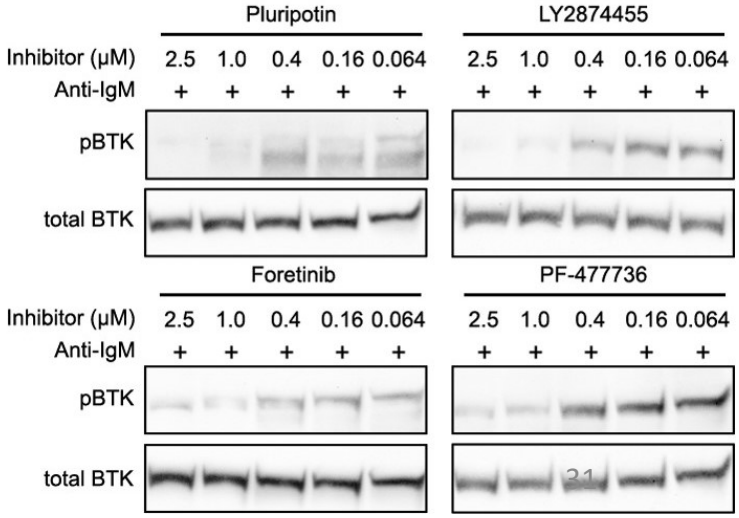


LY2874455

- 4 kinase inhibitors showed comparable BTK inhibition with ibrutinib.
- Pluripotin exhibited potent cellular inhibition of BTK phosphorylation at all concentrations.



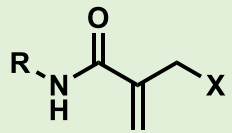




Conclusion

- **A new class of cysteine-targeting electrophiles for targeted covalent inhibitors**

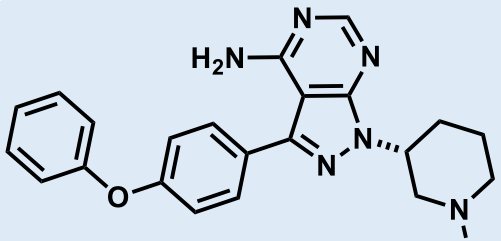
α-substituted methacrylamide



X = NR_2, OAr, OAc, OCOR

- Predictable reactivity
- Late-stage installation without modification to core scaffold
- Ability to functionalize compounds as turn-on probes

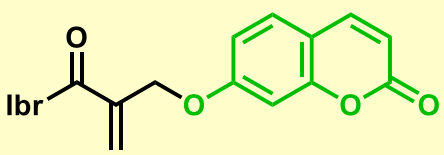
- **Ibrutinib-based methacrylamides deriviatives**



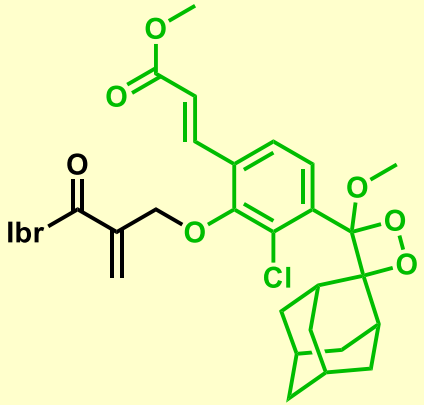
12 structures

- Most derivatives showed comparable BTK inhibiting activity with ibrutinib
- 2 derivatives with much higher selectivity over off-targets were found

- **Covalent ligand directed release chemistry**



turn-on fluorescent probe



turn-on chemiluminescent probe

- Besides fluorophores, a wide scope of compatible leaving group functionalities is supposed
- Tool for high-throughput screening on potent BTK inhibitors