Evaluation of covalent inhibition potency:
From IC_{50} to k_{inact}/K_I and beyond

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26 Sep 2024

Table of Contents

Kinetic mechanisms of covalent enzyme inhibition

"C2S": $E + I \underset{k_d}{\overset{k_a}{\rightleftharpoons}} E{\bullet}I \xrightarrow{k_f} EI$

"C2F": $E + I \overset{K_i}{\rightleftharpoons} E{\bullet}I \xrightarrow{k_f} EI$

$K_i = k_d/k_a$

"C1": $E + I \xrightarrow{k_a} EI$

Measures of covalent inhibitory potency

- **Microscopic rate constants (from continuous real-time assays)**
  - Mechanism "C2S": k_{a}, k_{d}, k_{f}
  - Mechanism "C2F": k_{d}, k_{f}
    [Note: k_{a} is *fixed* at a "diffusion controlled" value]
  - Mechanism "C1": k_{a}
- **Derived kinetic constants**
  - Mechanisms "C2S" and "C2F"
    - $k_{\mathrm{inact}} = k_{\mathrm{f}}$
    - $K_{\mathrm{i}} = k_{\mathrm{d}}/k_{\mathrm{a}}$
    - $K_{\mathrm{I}} = (k_{\mathrm{d}} + k_{\mathrm{f}})/k_{\mathrm{a}}$
    - $k_{\mathrm{inact}}/K_{\mathrm{I}} = k_{\mathrm{a}}\, k_{\mathrm{f}}/(k_{\mathrm{d}} + k_{\mathrm{f}})$
  - Mechanism "C1"
    - $k_{\mathrm{inact}}/K_{\mathrm{I}} = k_{\mathrm{a}}$
- **The IC_{50} (from end-point assays)**

A close look at the covalent IC_{50}

- Multiple definitions of covalent IC_{50} in the literature
- Time-dependence of covalent IC_{50}
- Dependence of covalent IC_{50} on other experimental factors

Multiple definitions of covalent IC_{50} in the literature

Repeat at various inhibitor concentrations (including zero):

- **Method 1**: [1]
  - Incubate enzyme + covalent inhibitor for a specific duration of time
  - Stop the covalent reaction somehow
  - Add substrate to determine amount of residual free enzyme
  - Compute the initial reaction rate since substrate was was added
- **Method 2**: [2]
  - Incubate enzyme + covalent inhibitor + substrate for a specific duration of time
  - Stop the enzymatic reaction
  - Determine the amount of product

  Method 3: [3]
  - Incubate enzyme + covalent inhibitor for a specific duration of time
  - Add substrate
  - Continue incubating enzyme + inhibitor + substrate for another fixed time interval
  - Stop the enzymatic reaction *and* the covalent E + I reaction
  - Determine the amount of product

[1] Kitz & Wilson (1962)
[2] Krippendorff *et al.* (2009)
[3] Fassunke *et al.* (2018)

Covalent IC_{50} is by definition time-dependent

A simulation study to illustrate time-dependence of IC_{50}:

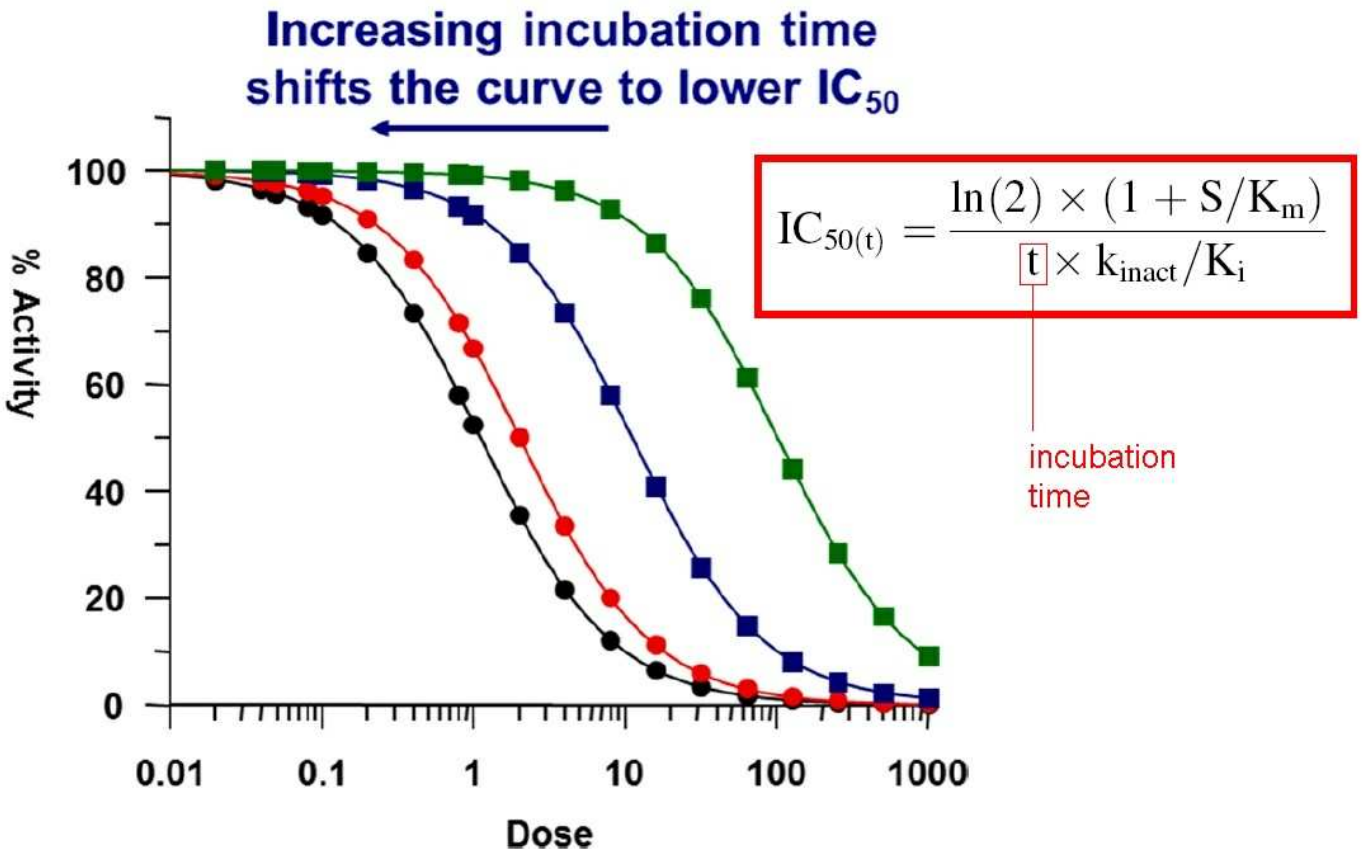


Figure 1. Dose response illustration of the time dependency of IC_{50} for covalent inhibitors (model data).

Thorarensen, A. *et al.* (2021) *Bioorg. Med. Chem.* **29**, 115865.

Time dependence of IC_{50}: An example

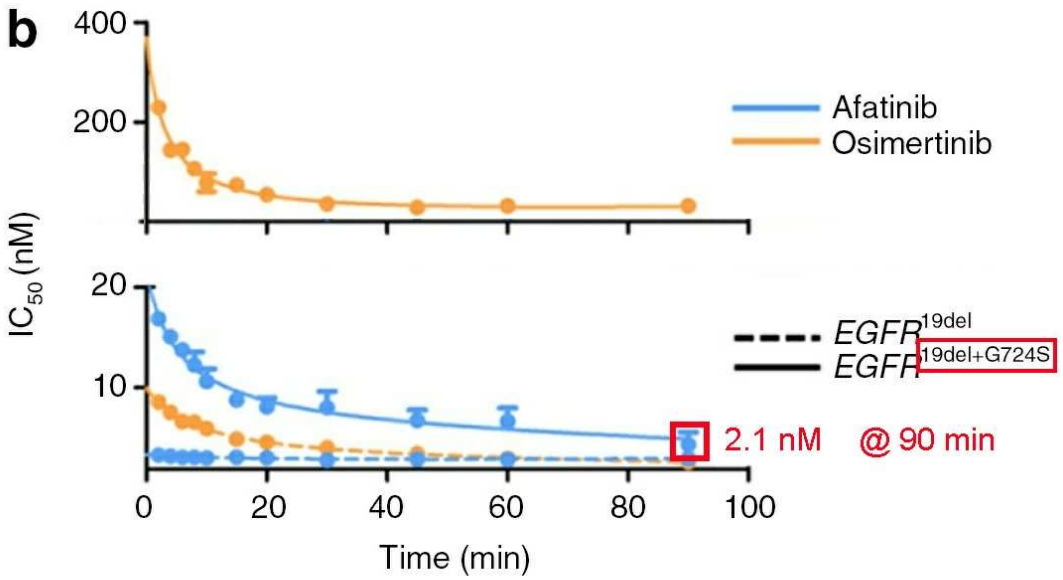


Fig. 5
b Time-dependent IC_{50}-determination of afatinib and osimertinib

Supplementary Table 3. Overview of IC_{50}

compound	EGFR	IC_{50} [nM]
	WT	<1
	L858R	<1
afatinib	L858R+T790M	1.3 ± 0.1
	19del	<1
	19del+G724S	2.1 ± 1.0

Fassunke, J. *et al.* (2018) *Nature Commun.* **9**, 4655.

Time dependence of IC_{50}: The asymptotic value at $t \to \infty$

- As incubation time approaches infinity, all covalent inhibitors end up with the same IC_{50}!
- Let us figure out together what it is.

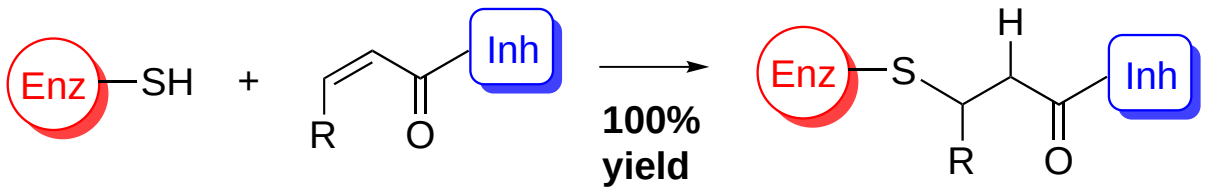
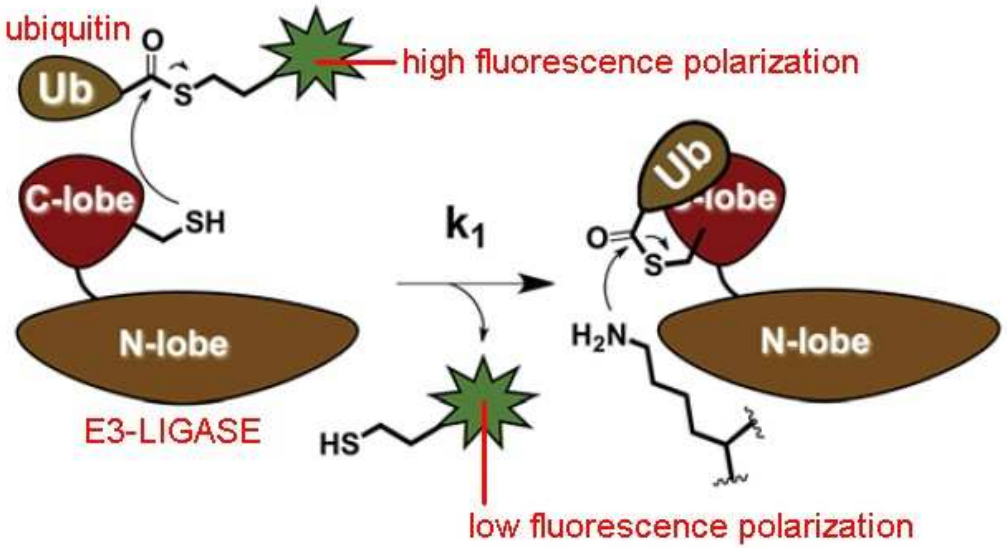


Table of Contents: E3 Ligase

- **I. Theory**: **Chemical and biochemical kinetics**
- **II. Applications**: **Three enzymology examples**
  - **Inhibition of an E3 ligase**: A clear **failure** of the IC_{50} method.
  - **Inhibition of the JAK3 kinase**: Power of the IC_{50} method is open to discussion.
  - **Inhibition of an EGFR mutant**: Beyond the $k_{\mathrm{inact}}/K_{\mathrm{I}}$.
- **III. Software**: **DynaFit / DynaPlate automation package**
- **IV. Open Discussion**

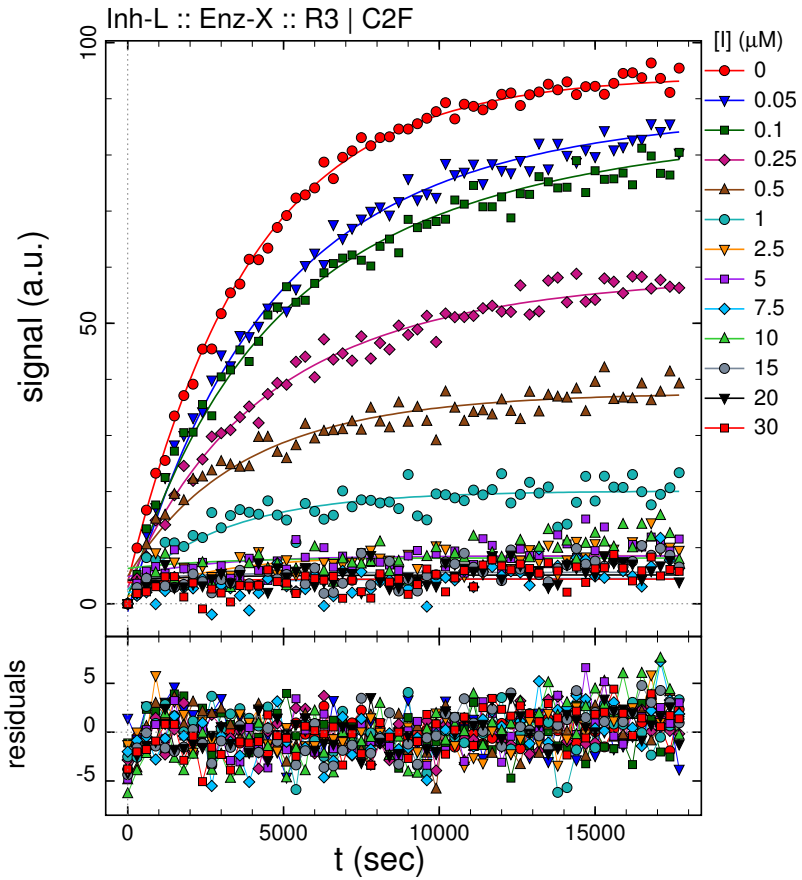
A very interesting E3 ligase assay



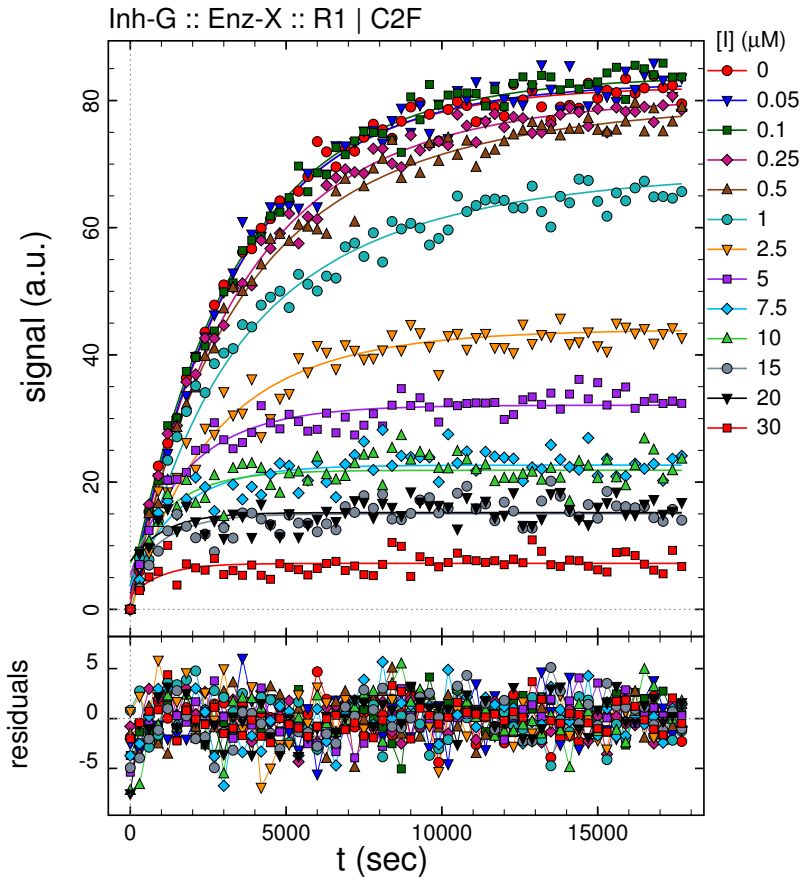
- An E3 ligase can be conveniently assayed in the absence of E1 and E2.
- Fluorescence polarization decreases as the fluorophore is detached from ubiquitin.

Krist, D. T. *et al.* (2016) *Chem. Sci.* **7**, 5587–5595

E3 ligase assay: Typical covalent inhibition datasets



“stronger” inhibitor



“weaker” inhibitor

- The observed decrease of fluorescence polarization was inverted for convenience.

E3 ligase assay: Reaction mechanism (DynaFit notation)

```
[mechanism]
   E + S ---> E + P      :    kaS
   E + I <==> E.I        :    ka      kd
   E.I ---> E-I          :    kf

[constants]
   kaS = 0.0059897
   ka = 100
   kd = {0.01, 0.1, 1, 10, 100} ??
   kf = {0.000001, 0.00001, 0.0001, 0.001, 0.01, 0.1} ??
```

- First-order substrate kinetics ($[S] << K_{\mathrm{m}}$).
- Two-step inhibitor binding ("rapid equilibrium" in the initial binding step).

E3 ligase assay: Mathematical model auto-generated by DynaFit

$$\frac{\mathrm{d}[\mathrm{E}]}{\mathrm{d}t} = -k_{\mathrm{a}}\,[\mathrm{E}][\mathrm{I}] + k_{\mathrm{d}}\,[\mathrm{E.I}] \tag{1}$$

$$\frac{\mathrm{d}[\mathrm{S}]}{\mathrm{d}t} = -k_{\mathrm{aS}}\,[\mathrm{E}][\mathrm{S}] \tag{2}$$

$$\frac{\mathrm{d}[\mathrm{P}]}{\mathrm{d}t} = +k_{\mathrm{aS}}\,[\mathrm{E}][\mathrm{S}] \tag{3}$$

$$\frac{\mathrm{d}[\mathrm{I}]}{\mathrm{d}t} = -k_{\mathrm{a}}\,[\mathrm{E}][\mathrm{I}] + k_{\mathrm{d}}\,[\mathrm{E.I}] \tag{4}$$

$$\frac{\mathrm{d}[\mathrm{E.I}]}{\mathrm{d}t} = +k_{\mathrm{a}}\,[\mathrm{E}][\mathrm{I}] - k_{\mathrm{d}}\,[\mathrm{E.I}] - k_{\mathrm{f}}\,[\mathrm{E.I}] \tag{5}$$

$$\frac{\mathrm{d}[\mathrm{E}-\mathrm{I}]}{\mathrm{d}t} = +k_{\mathrm{f}}\,[\mathrm{E.I}] \tag{6}$$

- This mathematical model was auto-generated by DynaFit software from `[mechanism]`.

E3 ligase assay: Model parameters

- Primary best-fit model parameters are the **optimized** microscopic rate constants:

k_d	dissociation rate constant
k_f	forward isomerization rate constant
k_a	association rate constant is a **fixed** model parameter

- Derived best-fit model parameters are the macroscopic kinetic constants:

$$K_\mathrm{i} = \frac{k_\mathrm{d}}{k_\mathrm{a}} \tag{7}$$

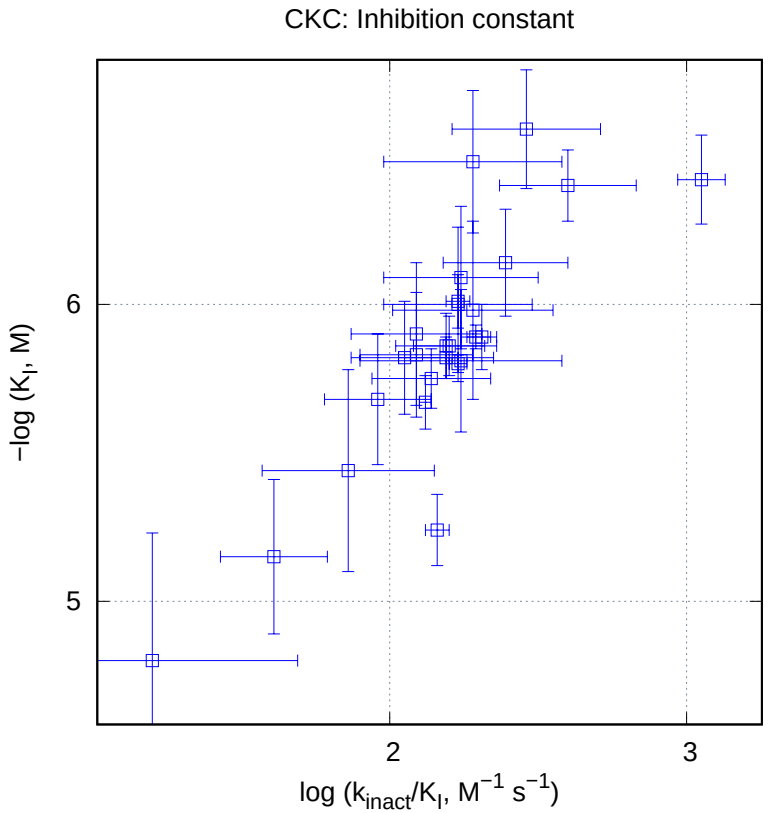
$$K_\mathrm{I} = \frac{k_\mathrm{d} + k_\mathrm{f}}{k_\mathrm{a}} \tag{8}$$

$$k_\mathrm{inact} = k_\mathrm{f} \tag{9}$$

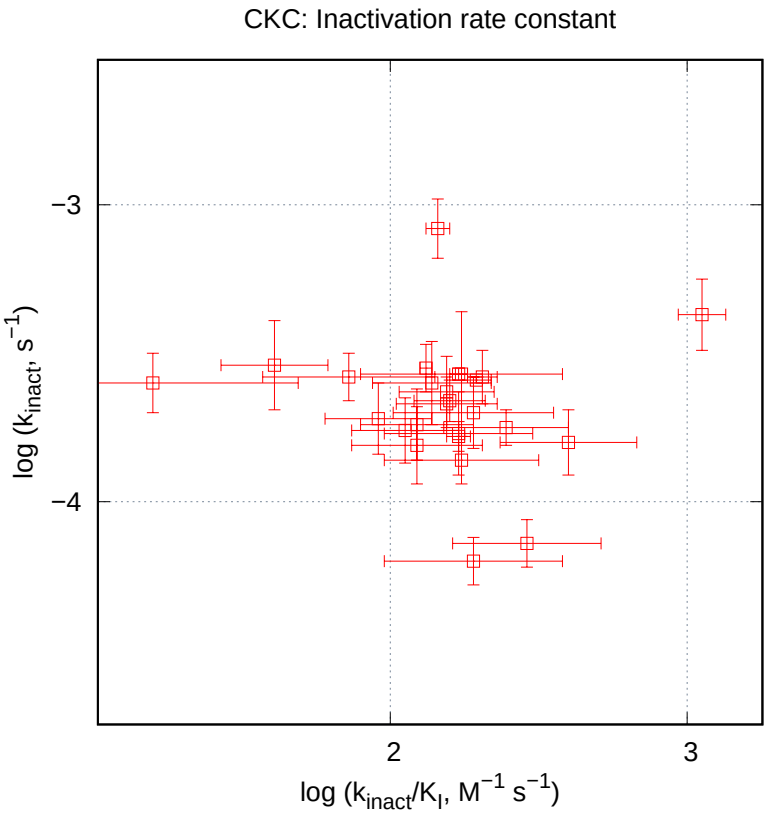
$$k_\mathrm{inact}/K_\mathrm{I} = k_\mathrm{f}\frac{k_\mathrm{a}}{k_\mathrm{d} + k_\mathrm{f}} \equiv k_\mathrm{eff} \tag{10}$$

  - "k_eff" a.k.a. $k_\mathrm{inact}/K_\mathrm{I}$ is the **covalent efficiency** constant.

E3 ligase assay: Covalent-kinetic correlation (CKC)



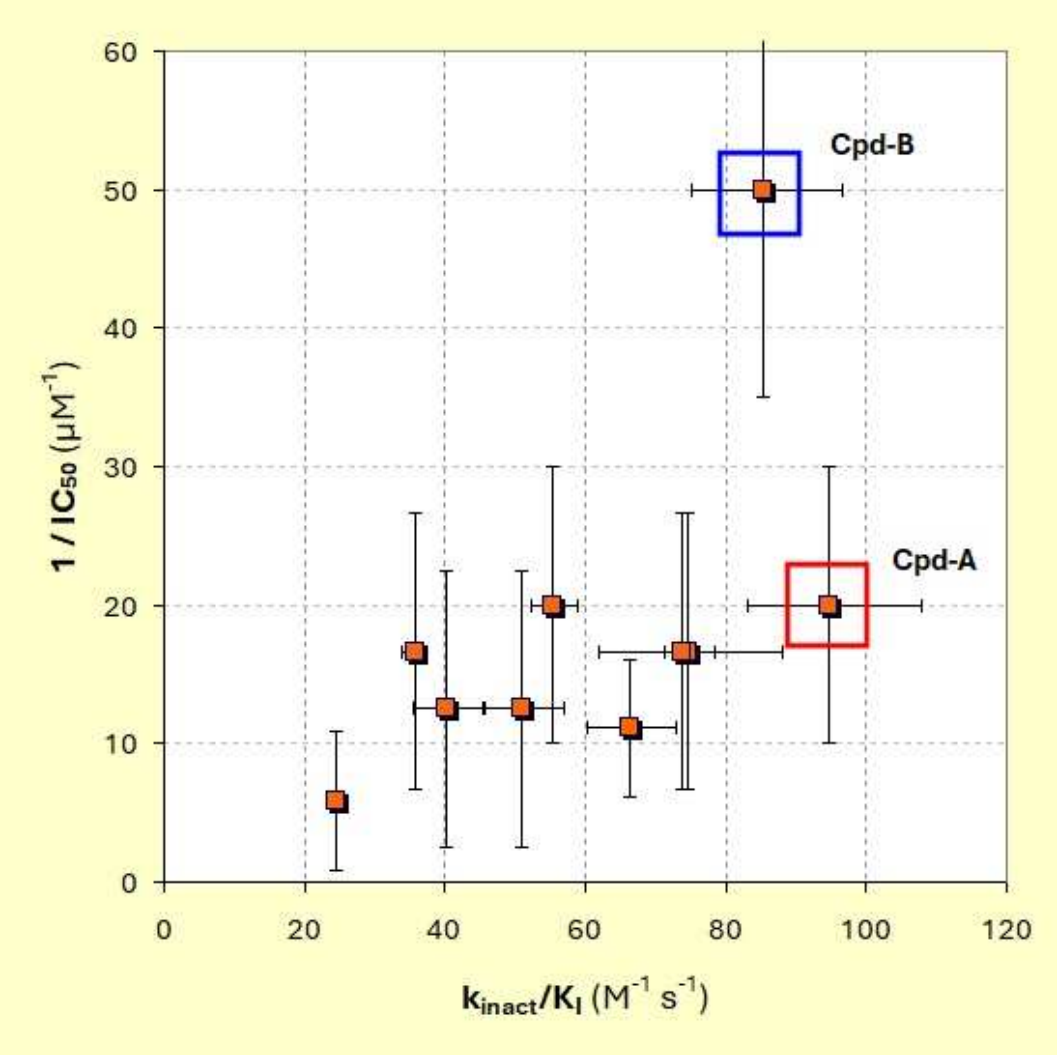
K_I **vs.** $k_\mathrm{inact}/K_\mathrm{I}$



k_inact **vs.** $k_\mathrm{inact}/K_\mathrm{I}$

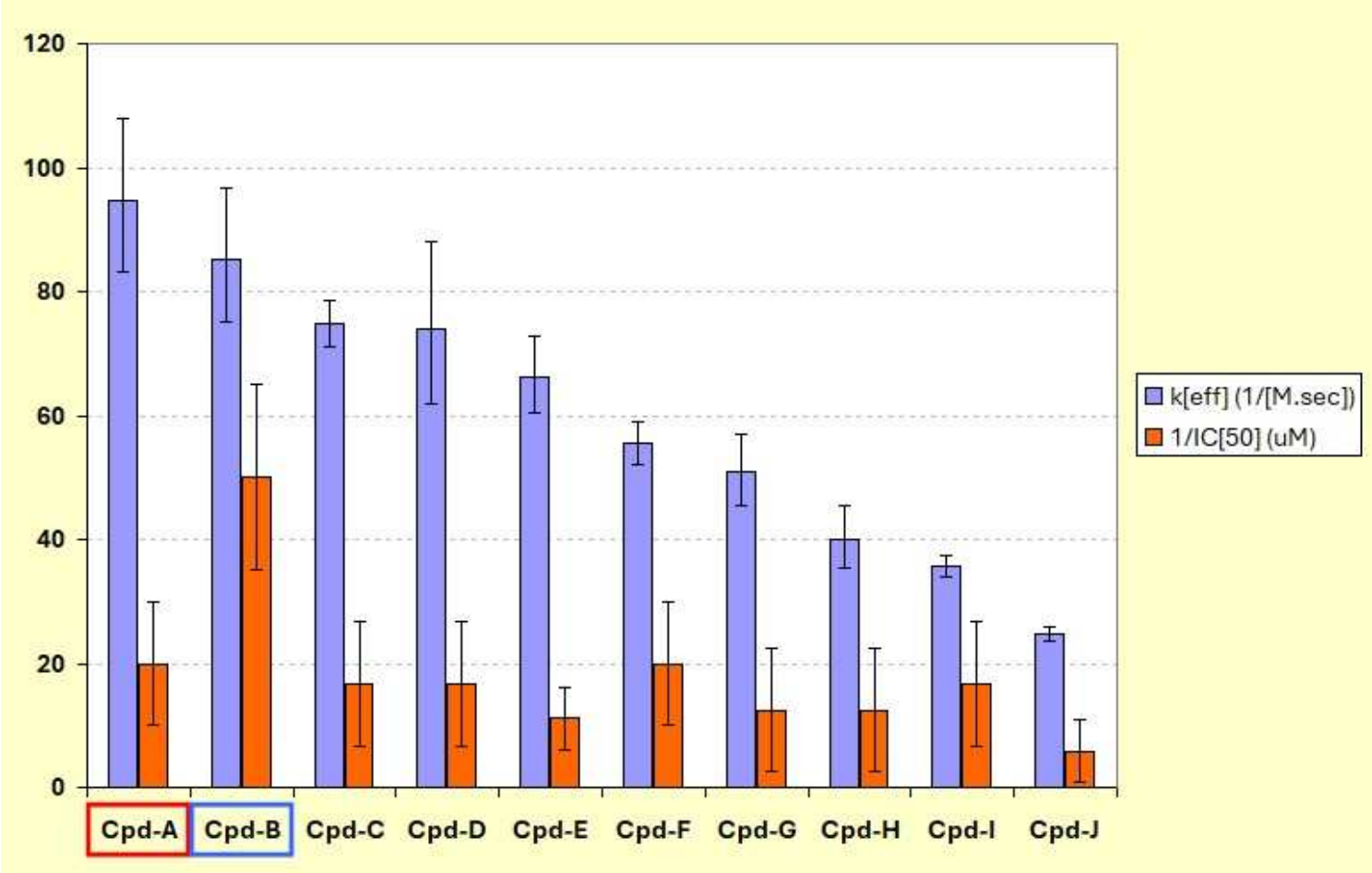
- The $k_\mathrm{inact}/K_\mathrm{I}$ value is determined mostly by the inhibitor's **binding affinity** (K_I).

E3 ligase assay: $k_{\mathrm{inact}}/K_{\mathrm{I}}$ vs. IC_{50} correlation



- The IC_{50} values were determined in a separate end-point (not "continuous") assay.

E3 ligase assay: $k_{\mathrm{inact}}/K_{\mathrm{I}}$ vs. IC_{50} order of potency



- The wrong "best" compound (B as opposed to A) was picked out by the IC_{50} method.

E3 ligase assay: Summary and conclusions

- For a set of "highly active" E3 ligase inhibitors, nearly all IC_{50} values are essentially identical.
- However, the IC_{50} method **falsely** singles out 'B' as the a "stand-out" compound:

$$B \quad >> \quad A = C = D = E = F = G = H = I = J$$

- In contrast, the k_{eff} values are much more distinct, which allows proper ranking.
- By the more accurate k_{eff} method, compound 'A' is most potent, closely followed 'B':

$$A \geq B \quad > \quad C \geq D \geq E \quad > \quad F \geq G \quad > \quad H \geq I \quad > \quad J$$

- In the specific case of E3 ligase inhibition, $k_{\mathrm{eff}} = k_{\mathrm{inact}}/K_{\mathrm{I}}$ **is clearly superior** to IC_{50}.

Table of Contents: JAK3

JAK3 kinase: "Advantages" of the IC_{50} ?

Bioorg. Med. Chem. 29 (2021) 115865

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

The advantages of describing covalent inhibitor in vitro potencies by IC_{50} at a fixed time point. IC_{50} determination of covalent inhibitors provides meaningful data to medicinal chemistry for SAR optimization

Atli Thorarensen [a,*], Paul Balbo [b], Mary E. Banker [c], Robert M. Czerwinski [b], Max Kuhn [d], Tristan S. Maurer [a], Jean-Baptiste Telliez [b], Fabien Vincent [c], Arthur J. Wittwer [b]

[a] *Medicine Design, Pfizer Worldwide R&D, 610 Main Street, Cambridge, MA 02139 USA*
[b] *Inflammation and Immunology, Pfizer Worldwide R&D, 610 Main Street, Cambridge, MA 02139 USA*
[c] *Medicine Design, Pfizer Worldwide R&D, Eastern Point Road, Groton, CT 06340 USA*
[d] *Research Statistics, Pfizer Worldwide R&D, Eastern Point Road, Groton, CT 06340 USA*

JAK3 kinase: Assumed inhibition mechanism

A. Thorarensen et al.

two-step binding mechanism:

$$E + I \underset{K_i}{\rightleftharpoons} E{\cdot}I \xrightarrow[k_{inact}]{} E{\cdot}I^*$$

Scheme 1.

- Two-step inhibitor binding ("rapid equilibrium" in the initial binding step).
- Identical to the mechanism also assumed for E3 ligase inhibition.

Thorarensen, A. *et al.* (2021) *Bioorg. Med. Chem.* **29**, 115865

JAK3 kinase: Reminder – time-dependence of IC_{50}

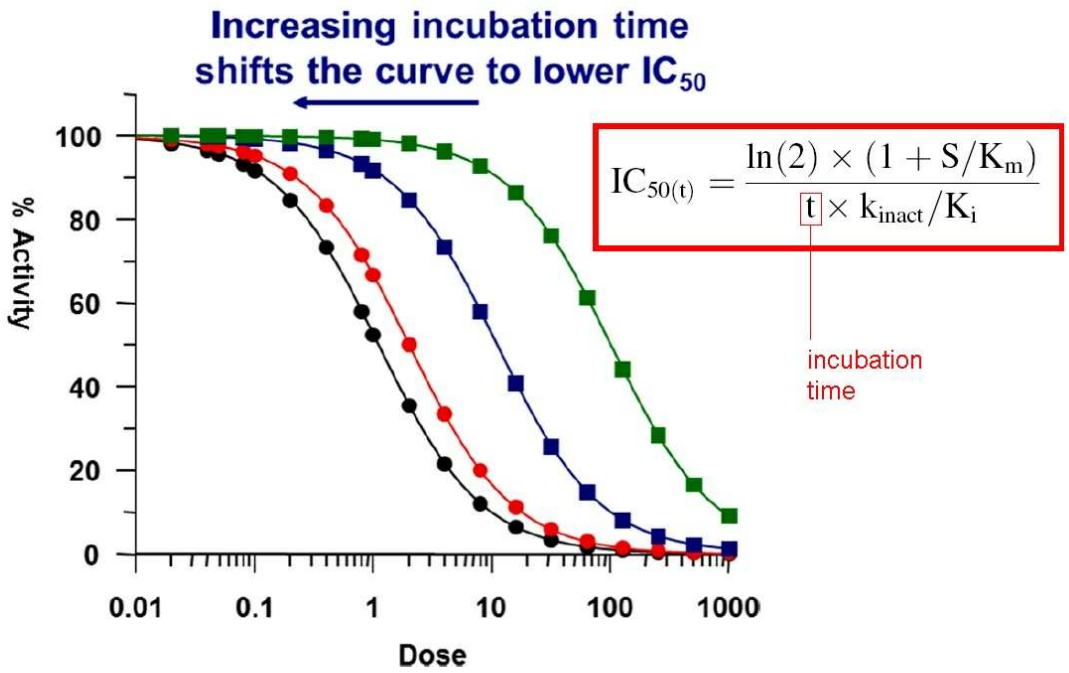


Figure 1. Dose response illustration of the time dependency of IC_{50} for covalent inhibitors (model data).

- *Article Abstract:* "The potency of covalent inhibitors is generally considered to be more accurately described by the **time-independent** kinetic parameter $k_{\mathrm{inact}}/K_{\mathrm{I}}$ rather than a by a simple IC_{50}, since the latter is a **time-dependent** parameter."

Thorarensen, A. *et al.* (2021) *Bioorg. Med. Chem.* **29**, 115865

JAK3 kinase: k_{inact}/K_I vs. IC_{50} correlation (as published)

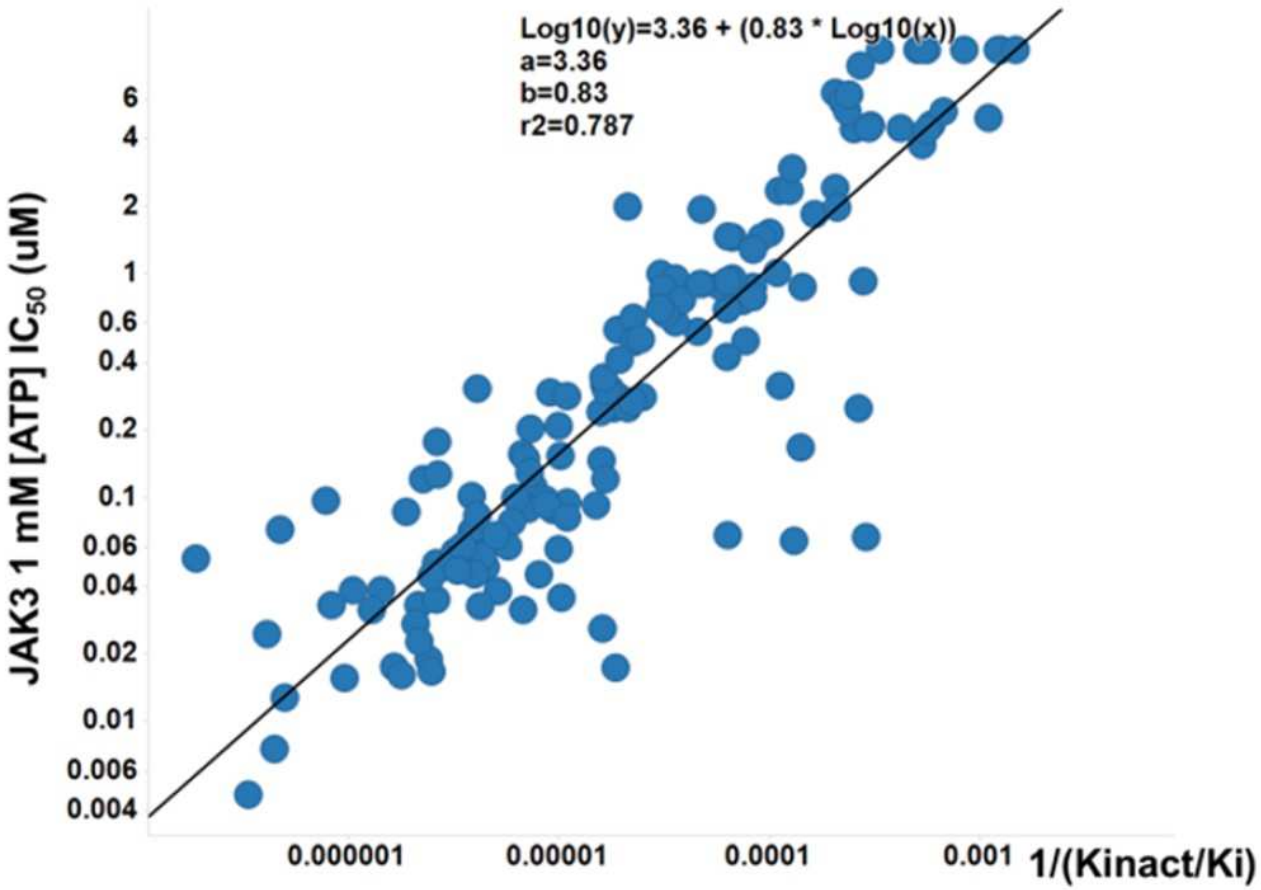
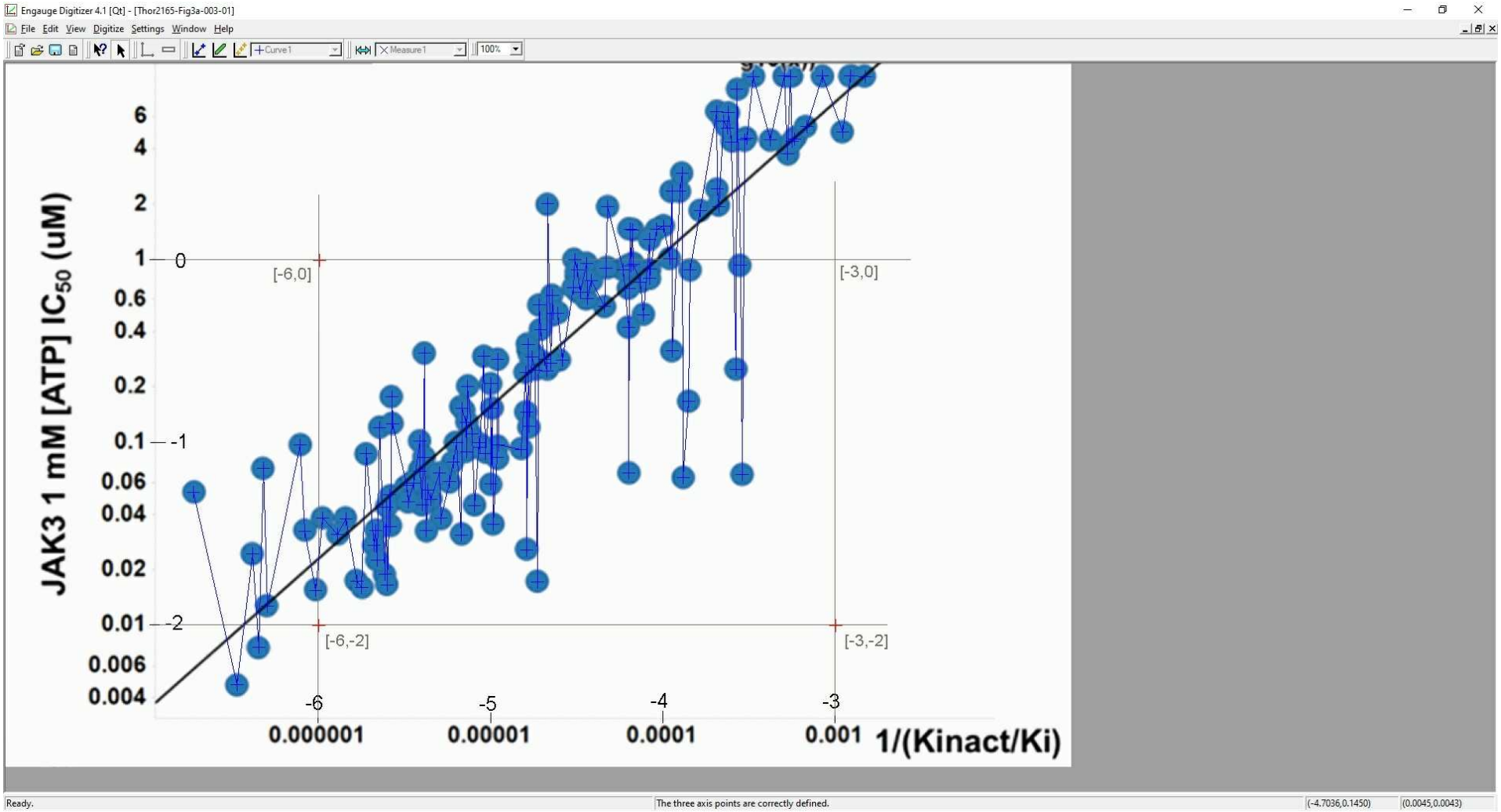


Figure 3. Relationship of k_{inact}/K_i with IC_{50} for JAK3 covalent inhibitors

Thorarensen, A. *et al.* (2021) *Bioorg. Med. Chem.* **29**, 115865

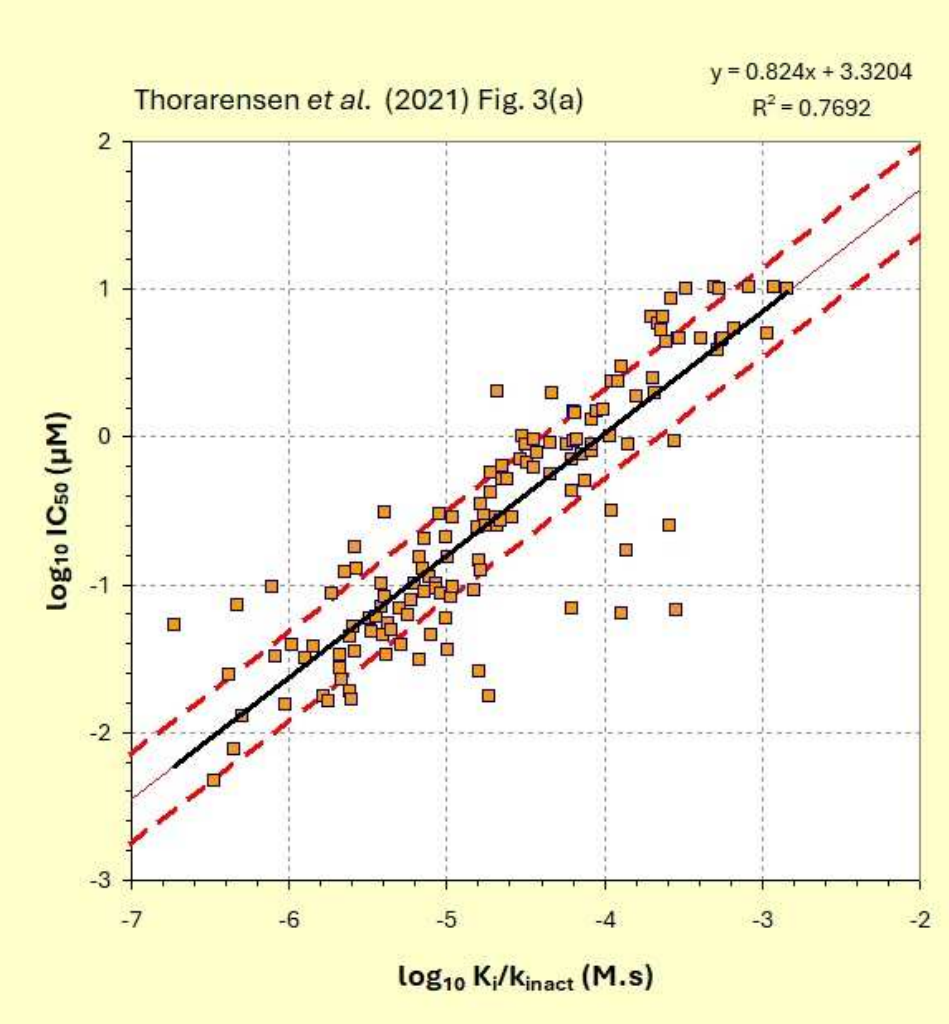
JAK3 kinase: $k_{\mathrm{inact}}/K_{\mathrm{I}}$ vs. IC_{50} correlation (digitized)

Digitization software: *Engauge ver. 4*



Thorarensen, A. *et al.* (2021) *Bioorg. Med. Chem.* **29**, 115865

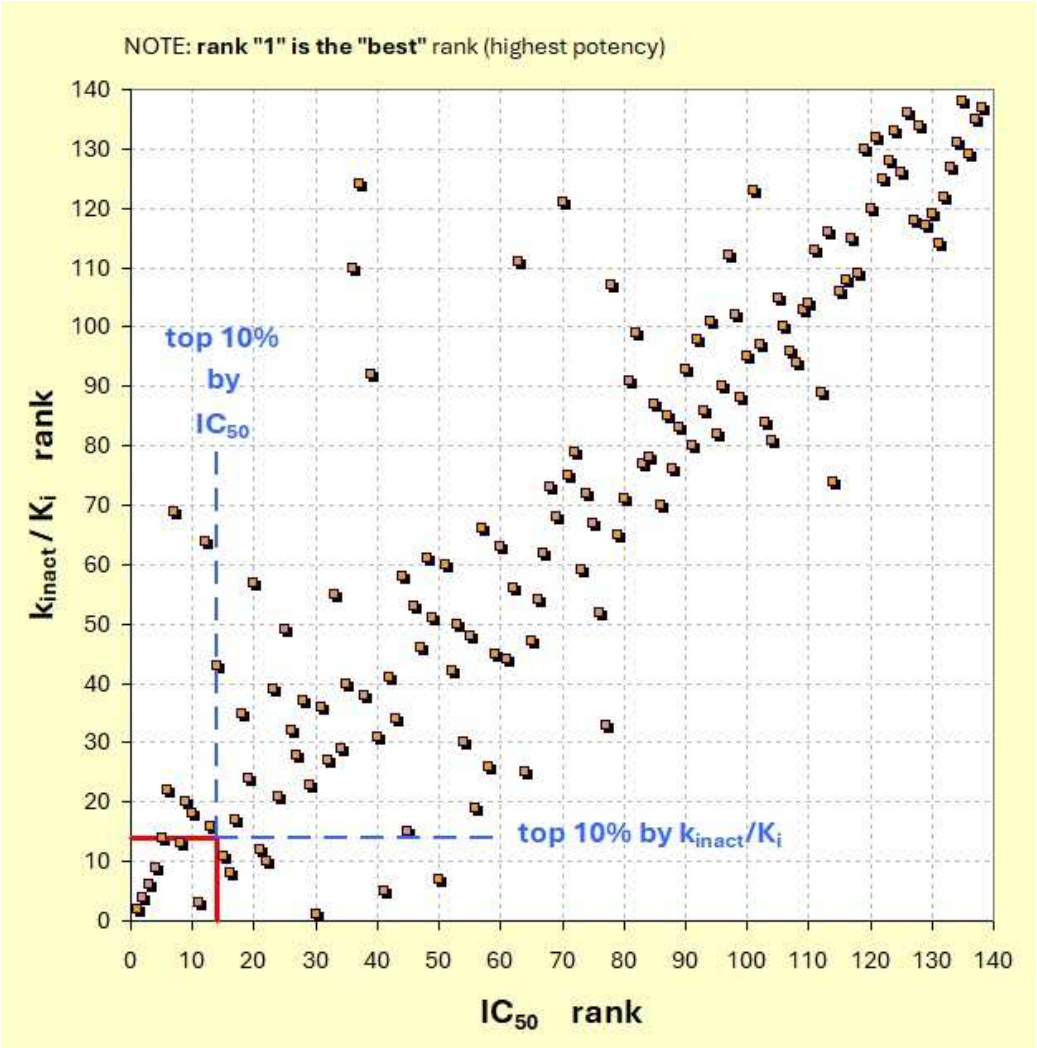
JAK3 kinase: $k_{\mathrm{inact}}/K_{\mathrm{I}}$ vs. IC_{50} correlation (digitized and analyzed)



- About **20 percent** of compounds are outside of **half-order-of-magnitude** band.
- The IC_{50} for **several** compounds is "off" by more than **two-orders of magnitude**.

Thorarensen, A. *et al.* (2021) *Bioorg. Med. Chem.* **29**, 115865

JAK3 kinase: $k_{\mathrm{inact}}/K_{\mathrm{I}}$ vs. IC_{50} potency rank



- The "top 10%" rule by IC_{50} is only about 50% efficient: it misses 1/2 of true "10%" hits.
- Even the "top 20%" rule by IC_{50} misses compounds ranked No. 1, 5, and 7 by $k_{\mathrm{inact}}/K_{\mathrm{I}}$.

JAK3 kinase: Summary and conclusions

Results

- The published $\log(IC_{50})$ vs. $\log(k_{inact}/K_I)$ **correlation** ($R^2 \approx 0.75$) looks impressive.
- However, a comparison of the corresponding **potency ranks** tells a different story:
- The IC_{50} method is about **50% efficient** in finding true hits ("top 10%" by k_{inact}/K_I).

Discussion

- An IC_{50} (a single-point assay) is definitely cheaper than k_{inact}/K_I (a continuous assay).
- The Big Question: Is this the usual "you get what you pay for" scenario?

Table of Contents: EGFR mutant

- **I. Theory**: **Chemical and biochemical kinetics**
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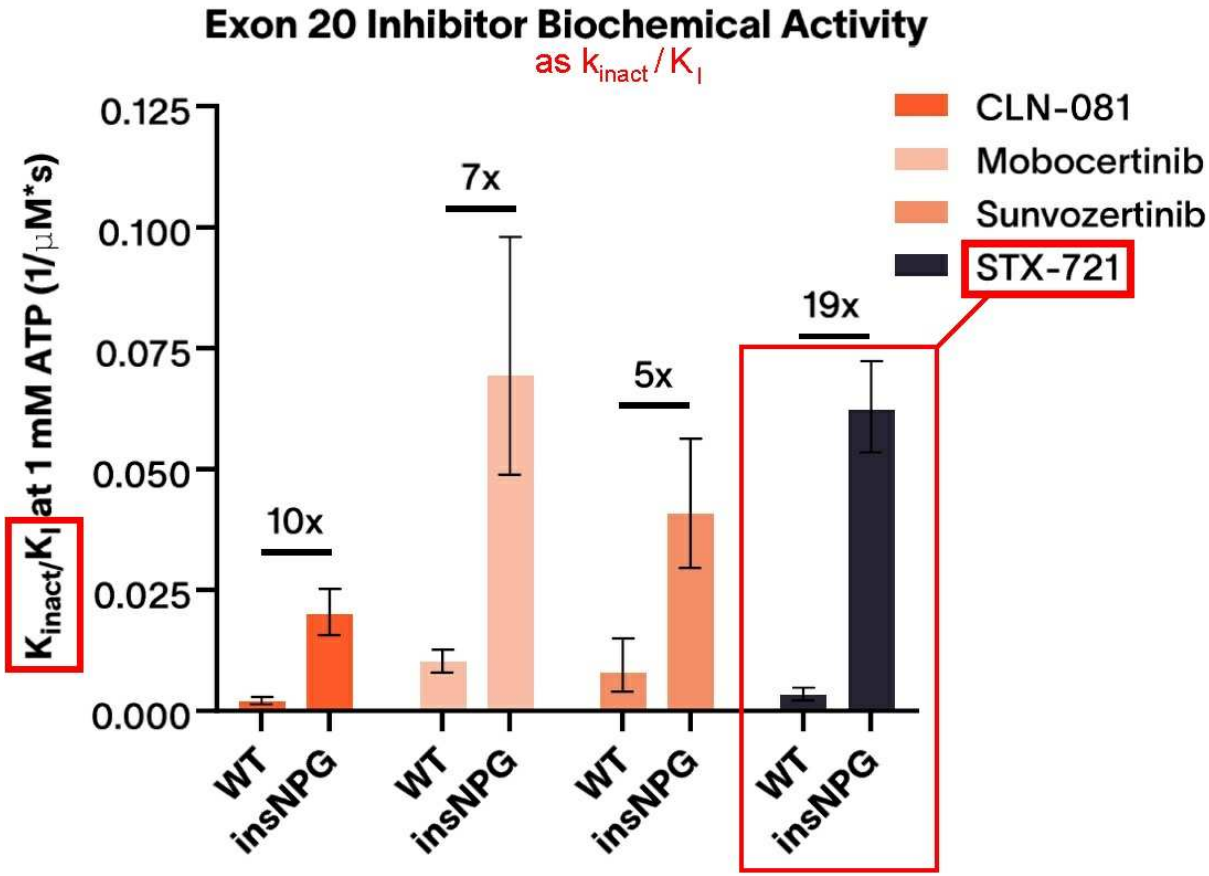
- **III. Software**: **DynaFit / DynaPlate automation package**
- **IV. Open Discussion**

EGFR exon 20 mutant: Covalent inhibitor STX-721 selectivity

Identification of STX-721, an EGFR exon 20 mutant inhibitor with superior selectivity and a potential best-in-class profile

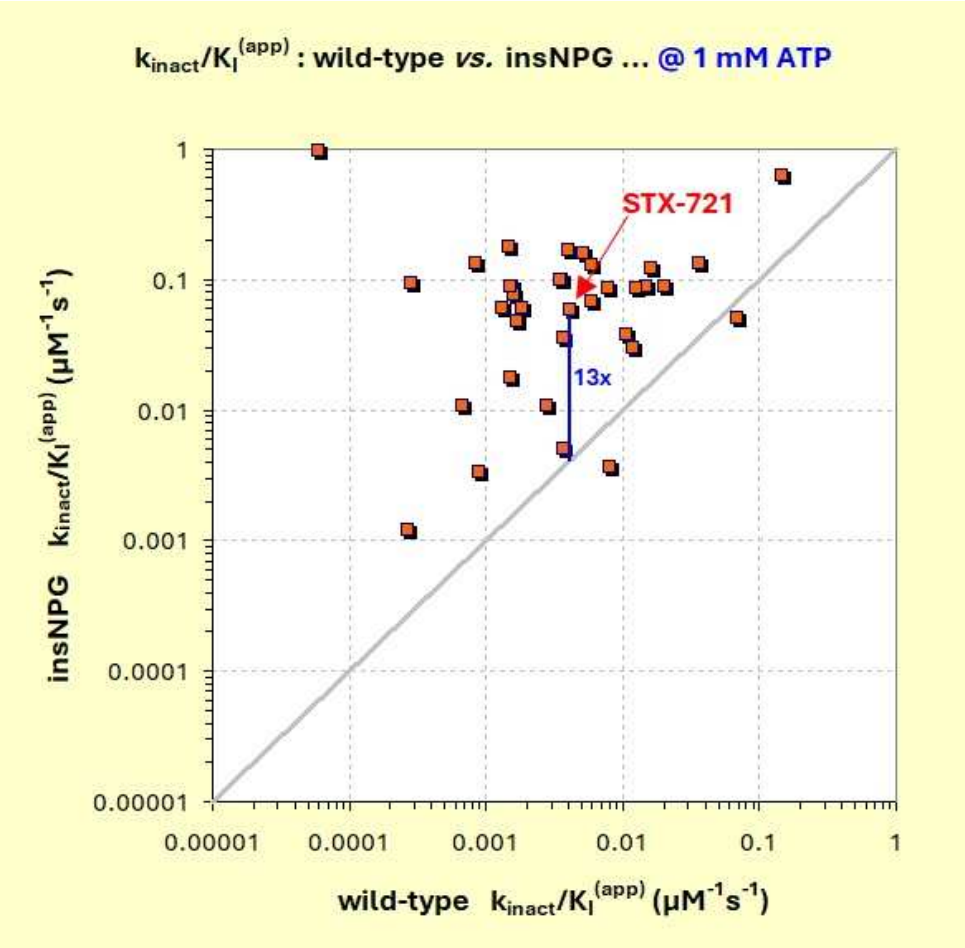
with Scorpion TX

Raymond A. Pagliarini[1], Benjamin C. Milgram[1], Deanna R. Borrelli[1], Erin O'Hearn[1], Michael R. Huff[1], Brendon Ladd[1], Takahiro Ito[1], Justine Bellier[3], Aaron N. Hata[3], Natasja Brooijmans[1], Philip Jonsson[1], Weixue Wang[1], Petr Kuzmic[2], Angel Guzman-Perez[1], Darrin D. Stuart[1]



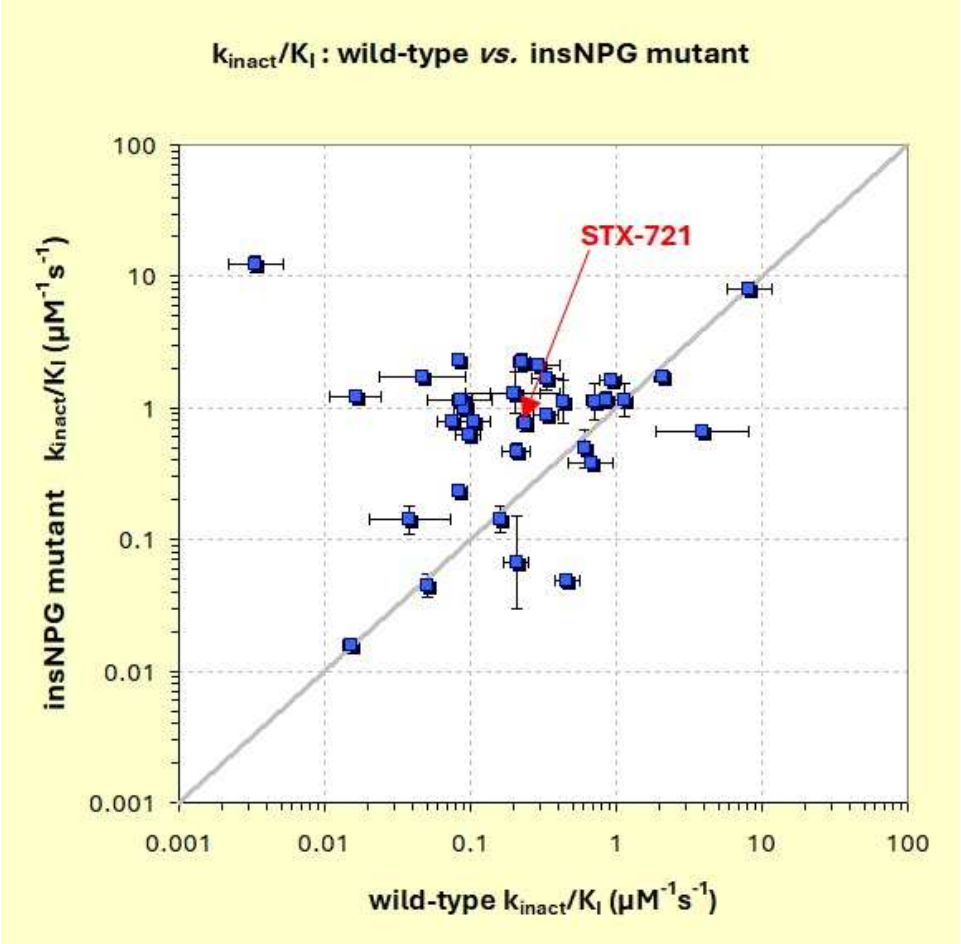
Pagliarini, R. A. *et al.* (2024) *Nature Cancer*, submitted.

STX-721 mutant / w.t. selectivity by "apparent" efficiency constant



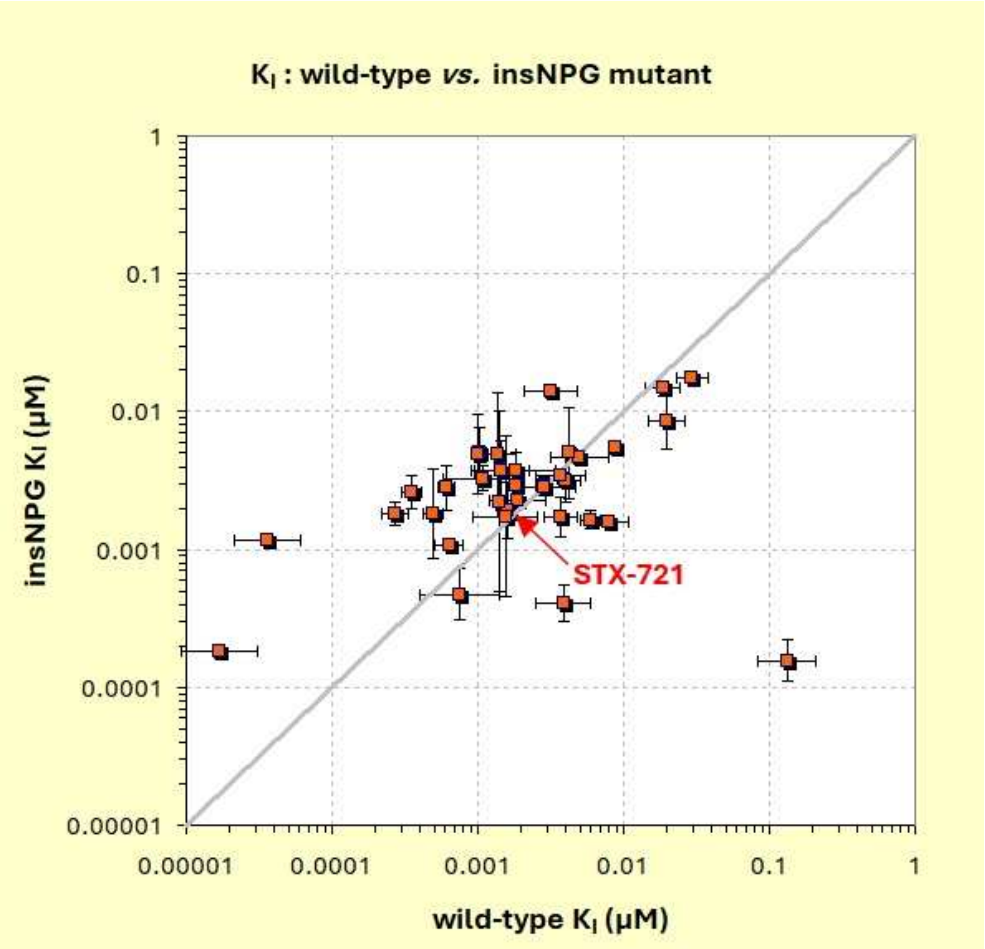
- "Apparent" efficiency constant: $k_{\mathrm{eff}}^{(app)} = k_{\mathrm{eff}}/(1 + [\mathrm{ATP}]/K_{\mathrm{m}}^{(ATP)})$
- Evaluated at $[\mathrm{ATP}] = 1.0$ mM, similar to intracellular environment.

STX-721 mutant / w.t. selectivity by "true" efficiency constant



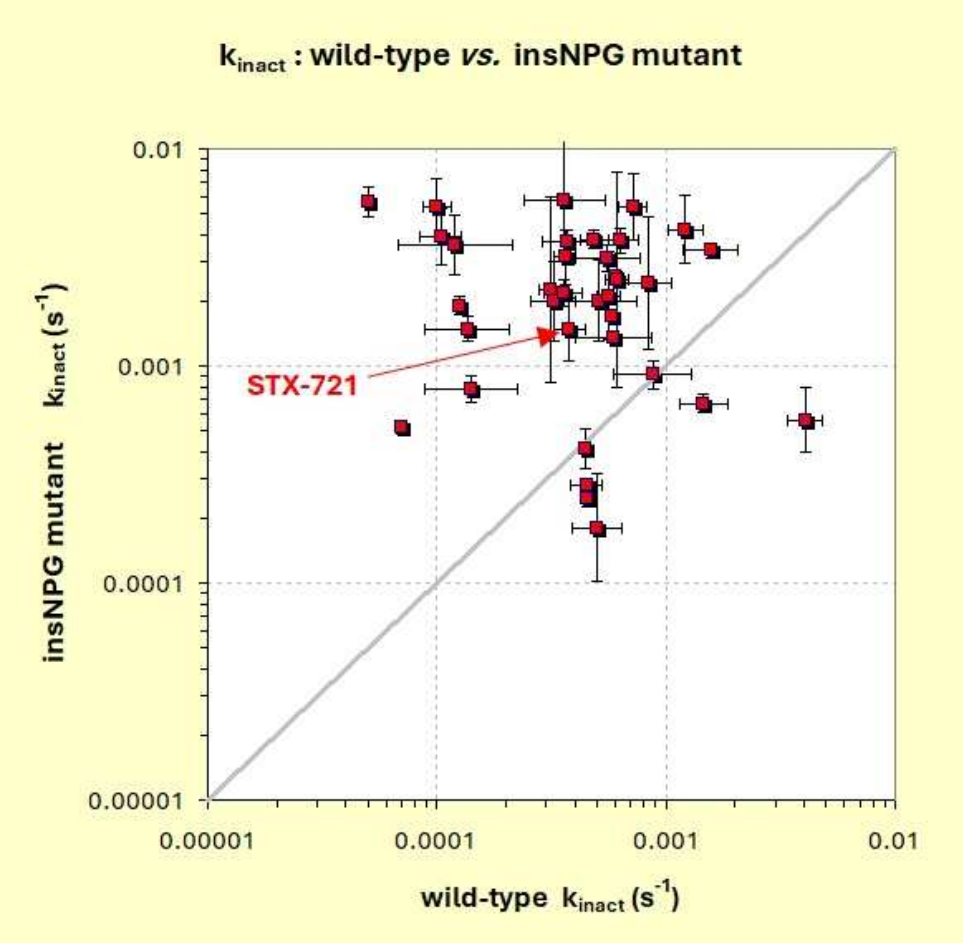
- "True" efficiency constant $k_{\mathrm{eff}} \equiv k_{\mathrm{inact}}/K_{\mathrm{I}}$ is uncorrected for cellular ATP.
- By this measure, STX-721 is still moderately selective (4x) for mutant over wild-type.

STX-721 mutant / w.t. selectivity by "true" inhibition constant



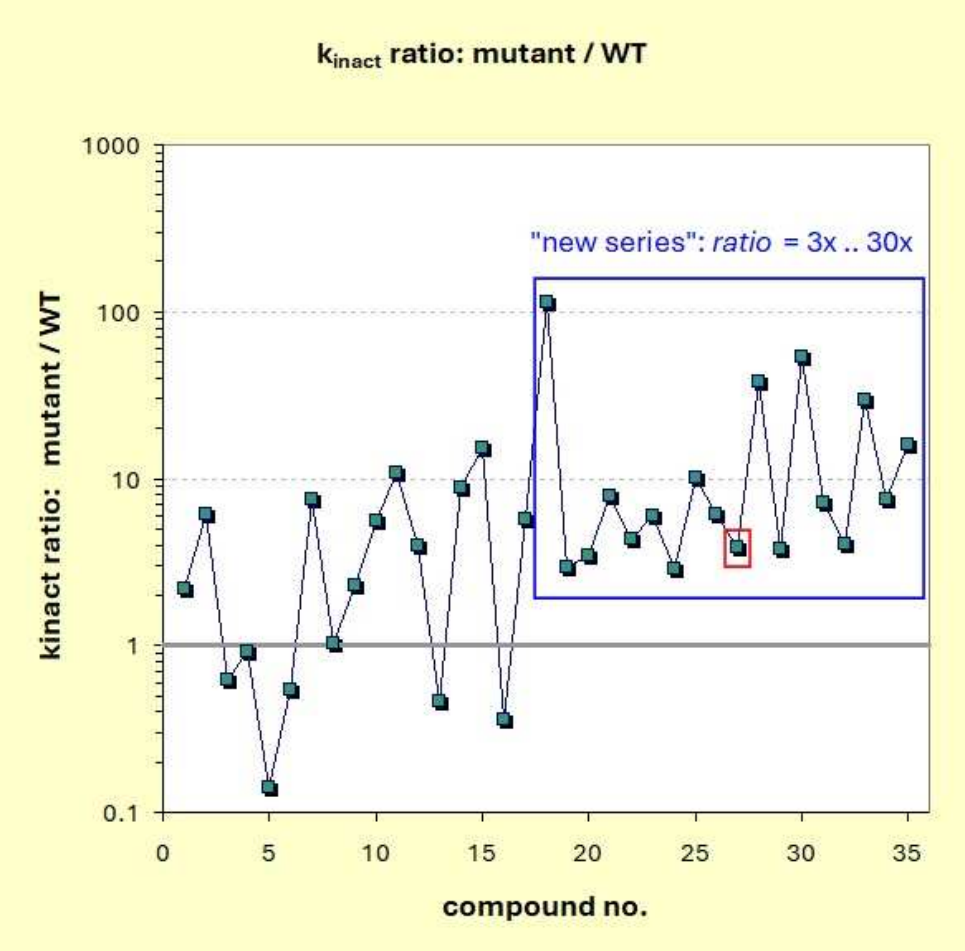
- "True" inhibition constants K_I (binding affinities) are identical for mutant and wild-type!

STX-721 mutant / w.t. selectivity by inactivation rate constant k_{inact}



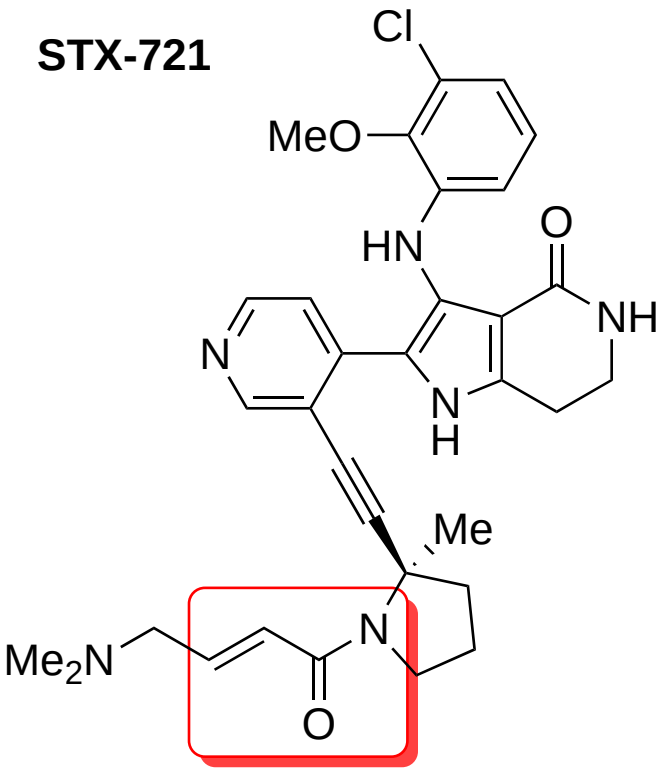
- **Selectivity** for mutant largely derives from **chemical reactivity** of the warhead.

STX-721 mutant / w.t.: inactivation rate constant k_{inact} ratios



- The fictitious "compound numbers" largely reflect the progress of this project over time.
- At some point, the team apparently stumbled upon a **mutant-selective warhead**.

STX-721 structure



- Mutant selectivity derives mostly from this warhead, or from its immediate surroundings.
- Binding affinity, due to the rest of the molecule, affects selectivity only to a minor degree.

Pagliarini, R. A. *et al.* (2024) *Nature Cancer*, submitted.

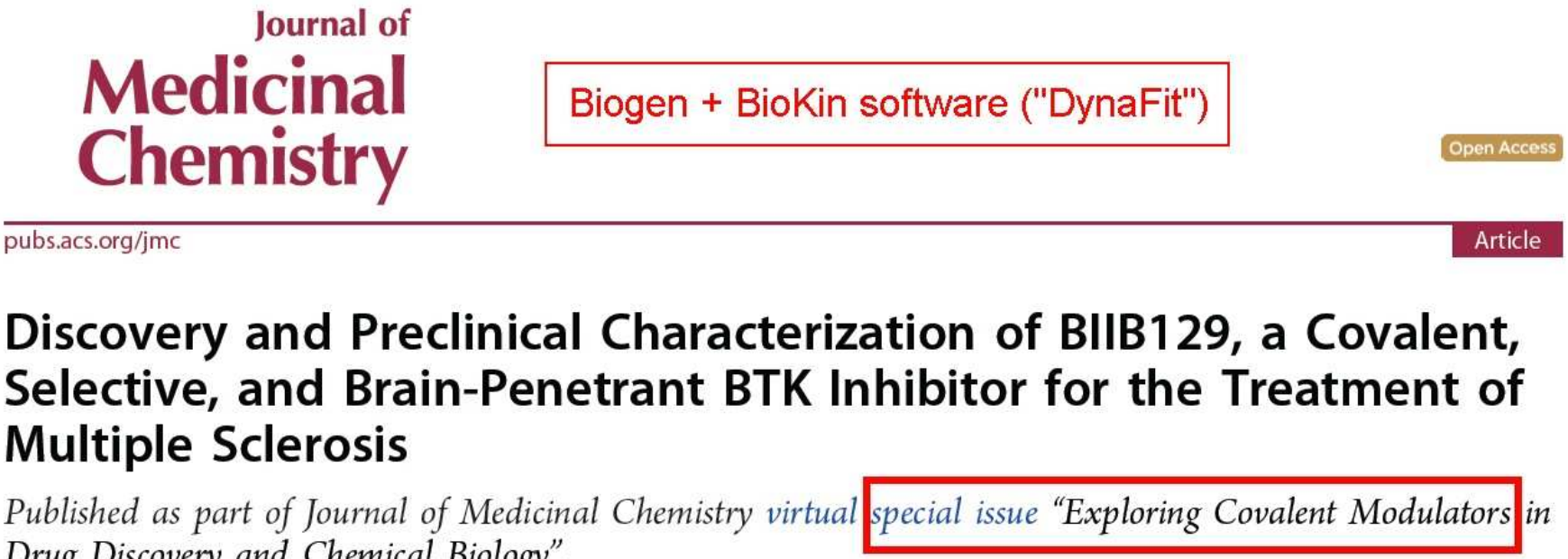
EGFR exon 20 mutant: Summary and conclusions

- The catalytic efficiency constant $k_{\mathrm{inact}}/K_{\mathrm{I}}$ does not alwys tell the whole story.
- For two-step covalent inhibitors, we should always look at k_{inact} and K_{I} separately.
- In most projects, k_{inact} stays about the same while K_{I} gets lower during optimization.
- However, in the case of the EGFR 770-insNPG-771 mutant, k_{inact} determines selectivity.

- In designing corporate databases storing covalent ligand potency, make room for all of these:
  - $k_{\mathrm{inact}}/K_{\mathrm{I}}$: the covalent efficiency constant
  - k_{inact}: the inactivation rate constant
  - K_{I}: the inhibition constant (binding affinity)

Table of Contents: E3 Ligase

EGFR exon 20 mutant: Covalent inhibitor STX-721 selectivity



- Biogen recently released all relevant biochemical kinetic data to BioKin for publication.
- A manuscript is being prepared by BioKin + Biogen, to describe all kinetics details.

Himmelbauer, M. K. *et al.* (2024) *J. Med. Chem.*, **67**, 8122-8140.