Covalent Inhibition Kinetics

Application to EGFR Kinase

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Covalent inhibition of protein kinases: Case study

ENZYME

Epidermal Growth Factor Receptor (EGFR) Kinase

9 **COVALENT** KINASE INHIBITORS

Gilotrif® (afatinib)
Dacomitinib
Neratinib
...
...

GOAL Determine basic biochemical characteristics of inhibitors:

(1) initial binding affinity: $\mathbf{K_i}$
(2) chemical reactivity: $\mathbf{k_{inact}}$

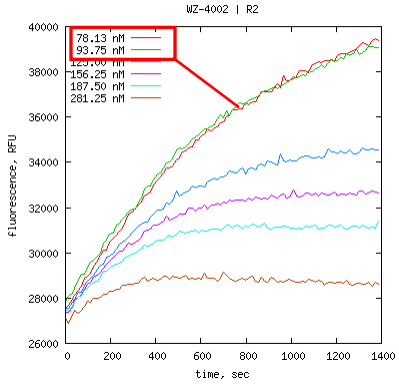
REFERENCE

Schwartz, P.; Kuzmic, P. *et al*. (2014)
Proc. Natl. Acad. Sci. USA. **111**, 173-178.

PRELIMINARIES:
ASSESSMENT OF RAW DATA

Reproducibility: Fixed vs. optimized concentrations

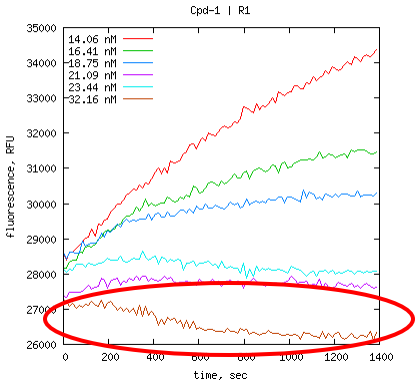
GLOBAL FIT OF **COMBINED** TRACES REQUIRES THAT CONCENTRATIONS ARE **CONSISTENT**



- Both of these inhibitor concentrations cannot be correct.
- Or can they ... ?
- We will treat inhibitor concentrations as adjustable parameters.

Outliers – Anomalous reaction progress

ABOUT **2% (10 / 400)** OF KINETIC TRACES IN THIS DATA STE ARE CLEARLY **ANOMALOUS**



Exclude this curve before analysis.

Optimal maximum inhibitor concentrations

IDENTICAL MAXIMUM CONCENTRATION **WOULD NOT WORK**

COMPOUND	$[I]_{max}$ nM
Afatinib	35.2
CL-387785	375.0
Cpd-2	62.5
Cpd-3	187.5
Cpd-4	7500.0
Cpd-5	1500.0
Dacomitinib	93.8
Neratinib	46.9
WZ-4002	187.5

- Maximum concentrations are based on preliminary experiments (IC_{50}).

Inhibitor vs. enzyme concentration: "Tight Binding" – Part 1

Compound	K_i^*, nM
CI-1033	1.6 ± 0.7
dacomitinib	18 ± 1
afatinib	4.4 ± 0.4
neratinib	4.0 ± 0.5
1	3.2 ± 0.5
CL-387785	180 ± 11
2	63 ± 5
3	84 ± 3
4	2200 ± 100
5	430 ± 30
WZ-4002	340 ± 20

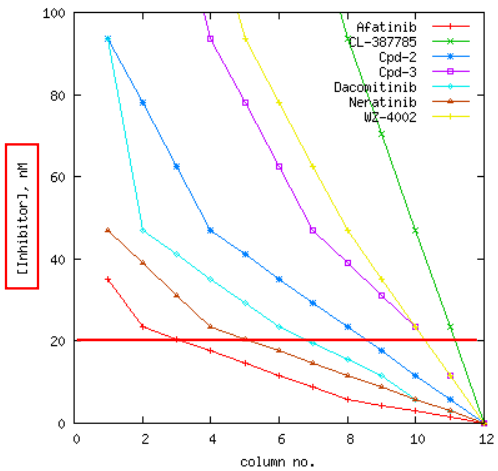
Table S 1: Apparent inhibition constants determined from the fit of *initial reaction rates* to the Morrison Eqn

- "Tight binding" is ***not*** a property of the **inhibitors**.
- "Tight binding" has to do with **assay conditions**.
- "TB" means that **$[E]_0 \cong K_i$ or even $[E]_0 > K_i$**

$[E]_0$ = 20 nM

Is $[E]_0$ is **very much lower** than K_i**?**

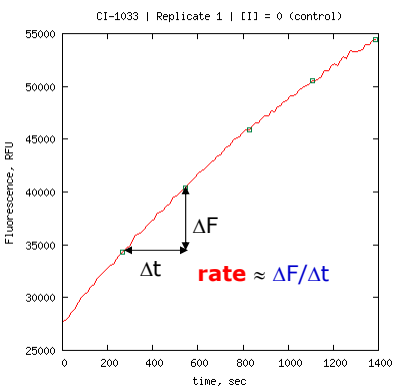
Inhibitor vs. enzyme concentration: "Tight Binding" – Part 2



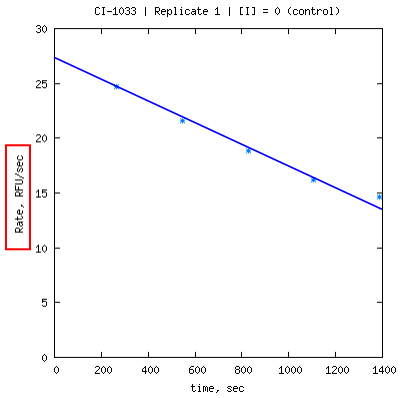
[Enzyme] = **20 nM**

- Inhibitor and enzyme concentrations are comparable.
- Inhibitor depletion does occur.

Substrate-only control: Linear or nonlinear?



Guess how much the slope (i.e., rate) changes between marked time points?



Reaction rate changes by **almost 50%** from start to finish: **NONLINEAR**.

Assessment of data: Implications for method of analysis

- Some curves will need to be excluded, preferably automatically.
- Concentrations will need to be treated as "unknown" parameters.

- Inhibition depletion does occur ("tight binding").
- Substrate depletion does occur (nonlinear control curve).

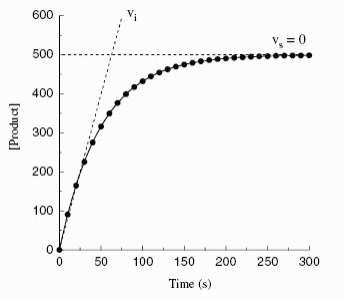
These facts will determine the choice of the mathematical model.

MATHEMATICAL MODELS:
ALGEBRAIC VS. DIFFERENTIAL EQUATIONS

"Traditional" method to analyze covalent inhibition data: Step 1

Copeland, R. (2013) *Evaluation of Enzyme Inhibitors in Drug Discovery* Second Edition, J. Wiley, New York, Chapter 9 (sect. 9.1)

Reaction progress at a given inhibitor concentration, $[I]_0$:

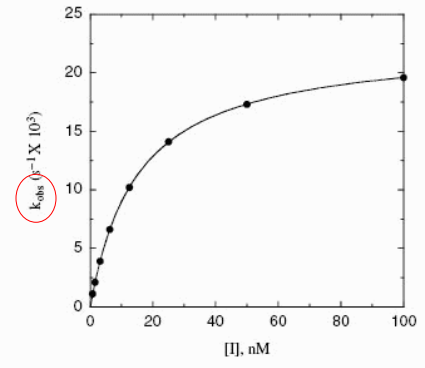


$$[P] = \frac{v_i}{k_{obs}}\left[1 - \exp(-k_{obs}\, t)\right]$$

Determine $\mathbf{k_{obs}}$ as a function of $[I]_0$

"Traditional" method to analyze covalent inhibition data: Step 2

Copeland, R. (2013) *Evaluation of Enzyme Inhibitors in Drug Discovery* Second Edition, J. Wiley, New York, Chapter 9 (sect. 9.1)



$$k_{obs} = k_{inact} \frac{[I]_0}{[I]_0 + K_i}$$

Nonlinear fit of **k_{obs}** values to determine **k_{inact}** and **K_i**

"Traditional" method: Underlying theoretical assumptions

1. **Linearity** of control curve

   Control progress curve ($[I]_0 = 0$) must be **strictly linear** over time.

2. **No** tight binding

   The noncovalent ***K_i*** value must be **very much higher than [enzyme]**.

- Both of these assumptions are violated for the inhibitors in our series.
- In fact, assumption #1 above is violated for all assays where $[S]_0 << K_M$.

- We **cannot use** the "traditional" method of kinetic analysis.

Alternate method: Differential equations (DynaFit software)

Kuzmic, P. (2009) "DynaFit – A software package for enzymology"
Meth. Enzymol. **467**, 247-280

INPUT TEXT:

```
[mechanism]
   E + S ---> E + P     :    ksub
   E + I <==> E.I       :    kaI    kdI
   E.I ---> E-I         :    kinact
```

INTERNALLY DERIVED MATHEMATICAL MODEL:

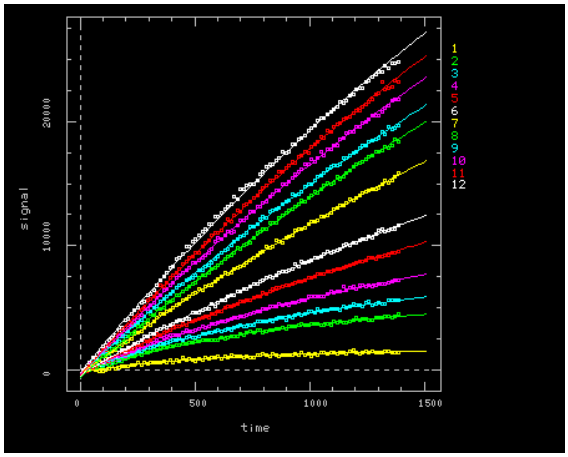
$$
\begin{aligned}
d[E]/dt &= -k_{sub}[E][S] + k_{sub}[E][S] - k_{aI}[E][I] + k_{dI}[E.I] \\
d[S]/dt &= -k_{sub}[E][S] \\
d[P]/dt &= +k_{sub}[E][S] \\
d[I]/dt &= -k_{aI}[E][I] + k_{dI}[E.I] \\
d[E.I]/dt &= +k_{aI}[E][I] - k_{dI}[E.I] - k_{inact}[E.I] \\
d[E\text{-}I]/dt &= +k_{inact}[E.I]
\end{aligned}
$$

system of differential equations

solved by using numerical methods

Differential equation method: Example – Afatinib: Data & model

DYNAFIT-GENERATED OUTPUT



"global fit"

combined progress curves analyzed together

Beechem, J. M. (1992) "Global analysis of biochemical and biophysical data"
Meth. Enzymol. **210**, 37-54.

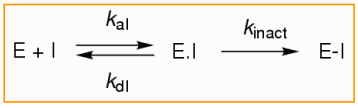
Differential equation method: Example – Afatinib: Parameters

DYNAFIT-GENERATED OUTPUT

Optimized Parameters

K_i = 0.0314 / **10** = **0.00314 µM**

No.	Par#Set	Initial	Final	Std. Error	CV (%)
#1	ksub	0.02	**0.0148823**	0.000279397	1.88
#2	kdI	0.06342	**0.0314187**	0.00188872	6.01
#3	kinact	0.01	**0.00204131**	0.00016957	8.31
#4	[E]	0.02	**0.0192718**	0.000358634	1.86



$\mathbf{K_i} = k_{dI} / k_{aI}$

k_{aI} = **10** $\mu M^{-1}s^{-1}$ ... assumed (fixed constant)

FINAL RESULTS: Afatinib – Replicate 1: $\mathbf{K_i}$ = 3.14 nM; $\mathbf{k_{inact}}$ = 0.00204 s^{-1}

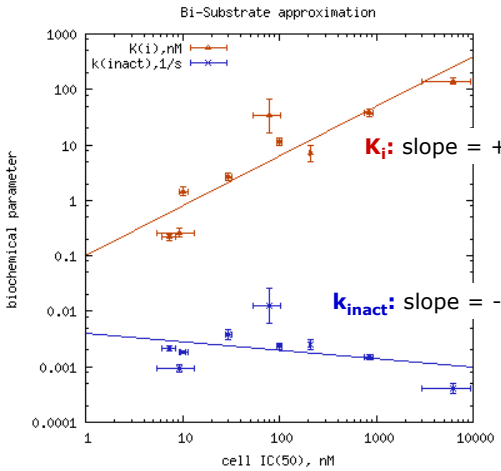
Differential equation method: Example – Afatinib: Reproducibility

EACH "REPLICATE" REPRESENT A **SEPARATE PLATE**

	k_{inact} s^{-1}	K_i nM	k_{inact} / K_i μM^{-1} s^{-1}
Replicate #1	**0.0020**	**3.1**	**10.4**
Replicate #2	**0.0021**	**3.1**	**10.5**
Replicate #3	**0.0025**	**4.0**	**9.9**

Reproducibility (**n=3**) of rate constants **5-15%** for **all compounds**.

Final results: Biochemical activity vs. Cellular potency



K_i: slope = +**0.90** **... initial binding**

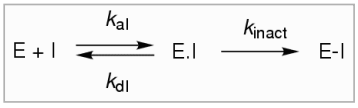
k_{inact}: slope = -**0.15** **... chemical reactivity**

Cellular potency correlates **strongly** with **binding**, but only weakly with reactivity.

CHECK UNDERLYING ASSUMPTIONS:
BIMOLECULAR ASSOCIATION RATE

Differential equation method: Example – Afatinib: Parameters

DYNAFIT-GENERATED OUTPUT



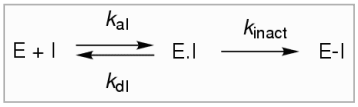
$K_i = k_{dI} / k_{aI}$

recall: we *assumed* this value

k_{aI} = **10** $\mu M^{-1}s^{-1}$ ... assumed (fixed constant)

Could the final result be skewed by making an **arbitrary assumption** about the magnitude of the association rate constant?

Varying assumed values of the association rate constant, k_{aI}



EXAMPLE: **Afatinib, Replicate #1/3**

ASSUMED	DETERMINED FROM DATA			
k_{aI}, $\mu M^{-1}s^{-1}$	k_{inact}, s^{-1}	k_{dI}, s^{-1}	K_i, nM	k_{inact}/K_i, $\mu M^{-1}s^{-1}$
10	0.0016	0.037	3.7	23.1
20	0.0016	0.074	3.7	23.1
40	0.0016	0.148	3.7	23.1

$K_i = k_{dI} / k_{aI}$

Effect of assumed association rate constant: Conclusions

The assumed value of the "on" rate constant

- **does** effect the best-fit value of the **dissociation** ("off") rate constant, k_{dI}.
- The fitted value of k_{dI} increases **proportionally** with the assumed value of k_{aI}.
- Therefore the best-fit value of the inhibition constant, **K_i, remains invariant**.
- The inactivation rate constant, k_{inact}, remains unaffected.

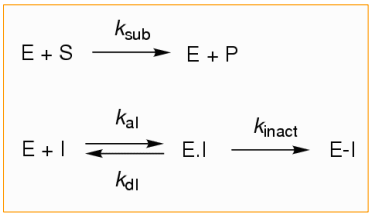
Assumptions about the "on" rate constant have **no effect** on the best-fit values of **k_{inact}, K_i,** and **k_{inact}/K_i.**

However, the dissociation ("off") rate constant remains **undefined** by this type of data.

CHECK UNDERLYING ASSUMPTIONS: SUBSTRATE MECHANISM

Substrate mechanism – "Hit and Run"

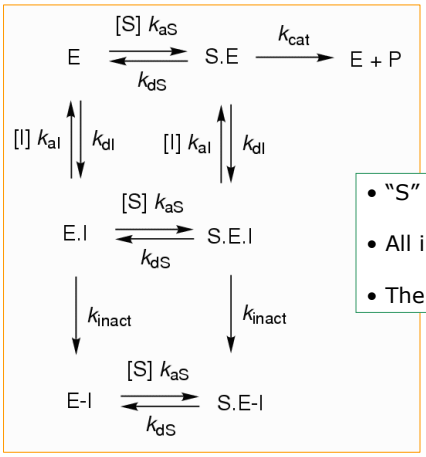
ASSUMING THAT THE **MICHAELIS COMPLEX** CONCENTRATION IS **EFFECTIVELY ZERO**



- Justified by assuming that $[S]_0 << K_M$
- In our experiments $K_M \geq$ **220 µM** and $[S]_0$ = **13 µM**
- The model was used in Schwartz *et al*. 2014 (PNAS)

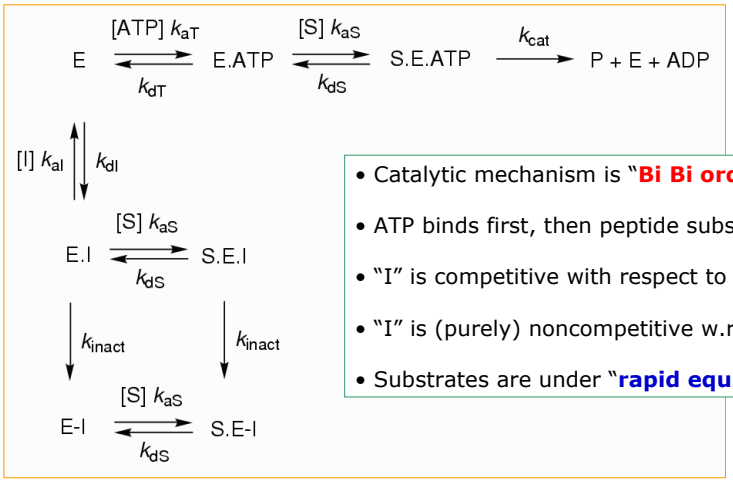
Substrate mechanism – Michaelis-Menten

ASSUMING THAT **ATP COMPETITION** CAN BE EXPRESSED THROUGH **"APPARENT" K_i**



- "S" is the peptide substrate
- All inhibitors are **ATP-competitive**
- Therefore they are **"S"-noncompetitive**

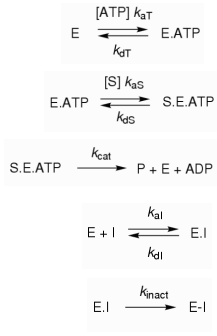
Substrate mechanism – Bi-Substrate



- Catalytic mechanism is "**Bi Bi ordered**"
- ATP binds first, then peptide substrate
- "I" is competitive with respect to ATP
- "I" is (purely) noncompetitive w.r.t. "S"
- Substrates are under "**rapid equilibrium**"

Substrate mechanism – "Bi-Substrate": DynaFit notation

MECHANISM:



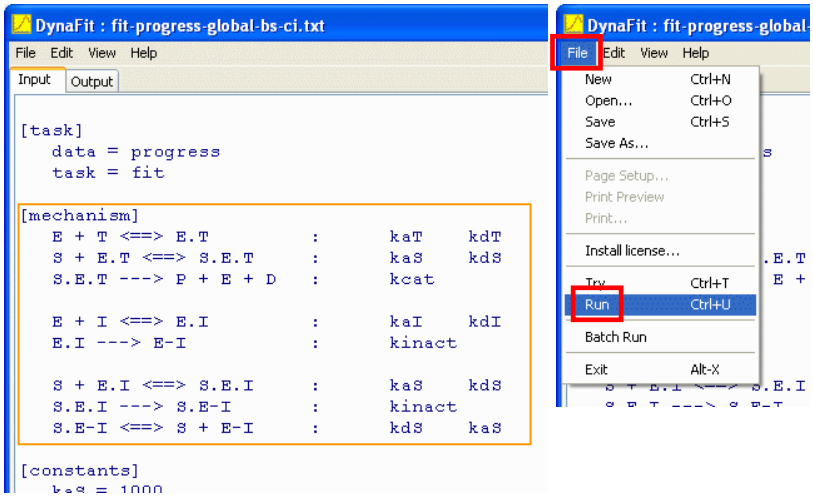
DYNAFIT INPUT:

```
[mechanism]

E + ATP <==> E.ATP          :   kaT    kdT

S + E.ATP <==> S.E.ATP      :   kaS    kdS

S.E.ATP ---> P + E + ADP    :   kcat

E + I <==> E.I              :   kaI    kdI

E.I ---> E-I                :   kinact
```

Similarly for the remaining steps in the mechanism.

Substrate mechanism – "Bi-Substrate": DynaFit notation

DYNAFIT INPUT WINDOW:



Presumed substrate mechanisms vs. k_{inact} and K_i

EXAMPLE: **AFATINIB, REPLICATE #1/3**

$$E + I \underset{k_{dI}}{\overset{k_{aI}}{\rightleftharpoons}} E.I \xrightarrow{k_{inact}} E\text{-}I$$

	FIXED k_{aI}, $\mu M^{-1}s^{-1}$	k_{dI}, s^{-1}	k_{inact}, s^{-1}	k_{dI}/k_{aI} K_i, nM
Hit-and-Run	**10**	**0.031**	**0.0019**	**3.1**
Michaelis-Menten	**10**	**0.033**	**0.0019**	**3.1**
Bisubstrate	**160**	**0.032**	**0.0019**	**0.19** = **3.1**/16

$[ATP]/K_{M,ATP}$ = 16

Substrate mechanism – Summary

1. Basic characteristic of inhibitors ($\mathbf{K_i}$, $\mathbf{k_{inact}}$) are essentially **independent** on the presumed substrate mechanism.

2. The inactivation rate constant ($\mathbf{k_{inact}}$) is **entirely invariant** across all three substrate mechanisms.

3. The initial binding affinity ($\mathbf{K_i}$) needs to be **corrected for ATP** competition in the case of "Hit and Run" and "Michaelis-Menten" mechanisms:

   - Hit-and-Run or Michaelis-Menten:
     Divide the measured $\mathbf{K_i^{app}}$ value by $\mathbf{[ATP]/K_{M,ATP}}$ to obtain true K_i

   - Bisubstrate:
     True K_i is obtained directly.

THE NEXT FRONTIER:
MICROSCOPIC "ON" AND "OFF" RATE CONSTANTS

Confidence intervals for "on" / "off" rate constants: Method

- We cannot measure "on" and "off" rate constants *as such*.
- But can can estimate at least the **lower limits** of their confidence intervals.

METHOD: **"Likelihood profile"** a.k.a. **"Profile-*t*"** method

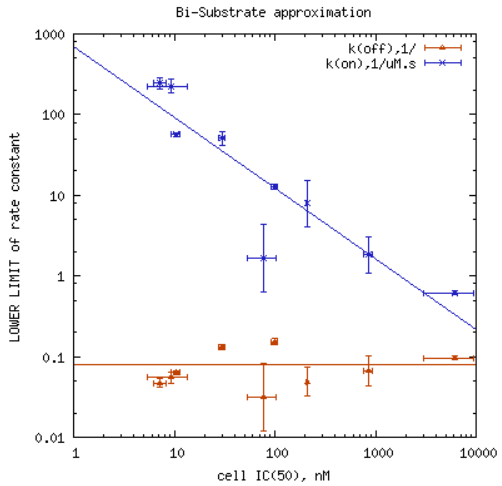
REFERENCES:

1. Watts, D.G. (1994)
"Parameter estimates from nonlinear models"
Methods in Enzymology, vol. **240**, pp. 23-36

2. Bates, D. M., and Watts, D. G. (1988)
Nonlinear Regression Analysis and its Applications
John Wiley, New York

sec. 6.1 (pp. 200-216) - two biochemical examples

Confidence intervals for "on" / "off" rate constants: Results



k_{on}: slope = **-0.88**
... association rate

k_{off}: slope = **~0.05**
... dissociation rate

Cell IC_{50} correlates strongly with **association rates**. Dissociation has no impact.

Cellular potency *vs.* upper limit of "residence time"

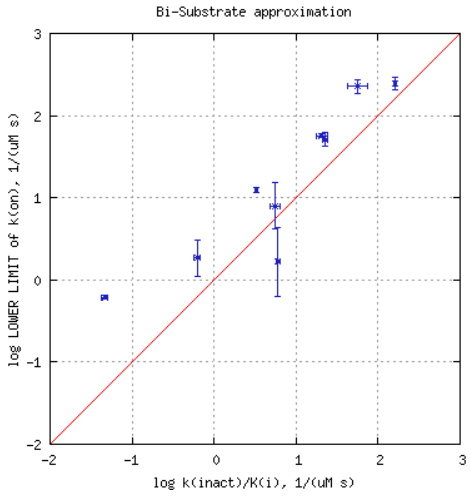
"Drug-receptor residence time": $\tau = 1 / k_{off}$

- **Lower** limit for "off" rate constant defines the **upper** limit for residence time.
- Both minimum k_{off} and **maximum τ is invariant** across our compound panel.
- However cellular IC_{50} varies by 3-4 orders of magnitude.

- This is unexpected in light of the "residence time" theory of drug potency:

Copeland, Pompliano & Meek (2006) *Nature Rev. Drug Disc.* **5**, 730
Tummino & Copeland (2008) *Biochemistry* **47**, 5481.
Copeland (2011) *Future Med. Chem.* **3**, 1491

Lower limit for the "on" rate constant vs. k_{inact}/K_i



k_{inact}/K_i from rapid-equilibrium model is a good "proxy" for minimal k_{on}.

Summary and conclusions

1. **EQUILIBRIUM BINDING AFFINITY:**

   Initial (non-covalent) binding seems more important for cell potency than chemical reactivity.

2. **BINDING DYNAMICS:**

   Association rates seem more important for cell potency than dissociation rates (i.e., "residence time").

3. $\mathbf{k_{inact} / K_i}$ (rapid-equilibrium) appears to be a good proxy for the lower limit of the "on" rate constant.

This work could **not** have been done using the "usual" data-analysis method:

- substrate depletion ($[S]_0 << K_M$)
- inhibitor depletion ($[I]_0 \sim [E]_0$)

➡ use **DynaFit** to analyze covalent inhibition data

Acknowledgments

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- Jim Solowiej

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