New Insights into Covalent Enzyme Inhibition

Application to Anti-Cancer Drug Design

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December 5, 2014
Brandeis University

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Synopsis

For a *particular* group of covalent (irreversible) protein kinase inhibitors:

- Cellular potency is driven mainly by the **initial noncovalent binding**.
- **Chemical reactivity** (covalent bond formation) plays only a minor role.
- Of the two components of initial binding:

  - the **association rate** constant has a dominant effect, but
  - the **dissociation rate** constant appears unimportant.

- These findings appear to contradict the widely accepted **"residence time"** hypothesis of drug potency.

REFERENCE

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

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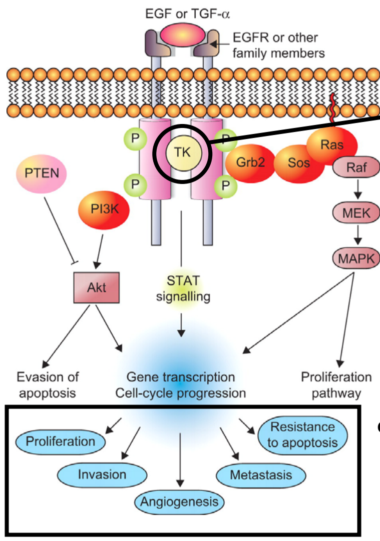
The target enzyme: Epidermal Growth Factor Receptor (EGFR)



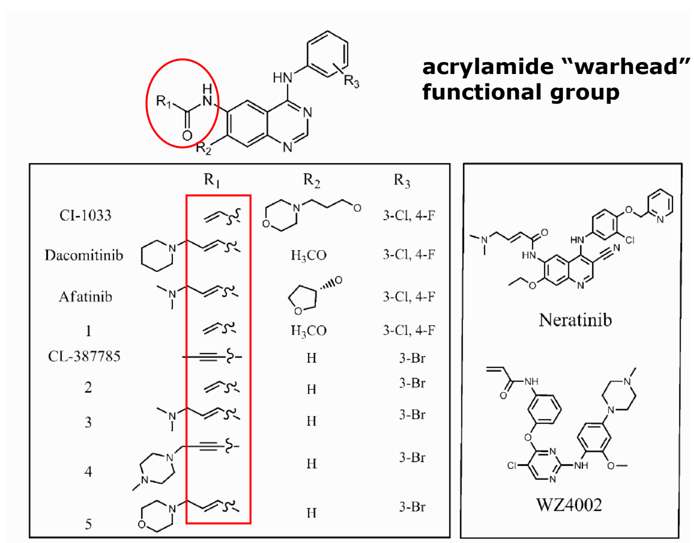
tyrosine kinase activity

kinase inhibitors act as anticancer therapeutics

cancer

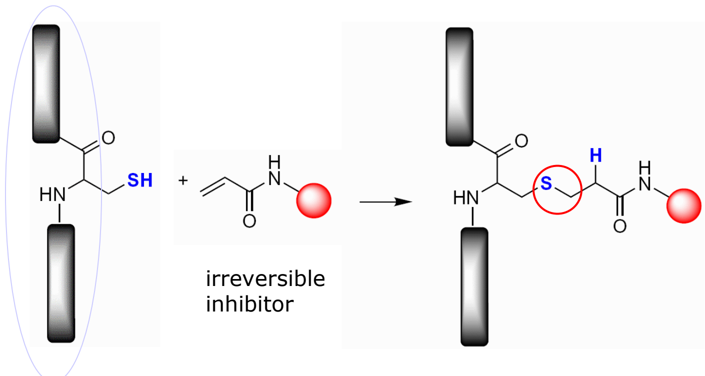
http://ersj.org.uk/content/33/6/1485.full

EGFR kinase inhibitors in the test panel



acrylamide "warhead" functional group

Covalent inhibitors of cancer-related enzymes: Mechanism

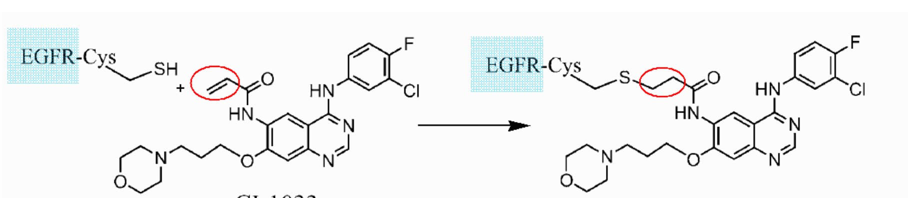


protein
chain

covalent
adduct

EGFR inhibition by covalent drugs: Example

Michael addition of a cysteine –SH group



CI-1033

Canertinib (CI-1033): experimental cancer drug candidate

Two steps: 1. non-covalent binding, 2. inactivation

binding affinity

$K_i = k_{off} / k_{on}$

chemical reactivity

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E{\bullet}I \xrightarrow{k_{inact}} E{\sim}J$$

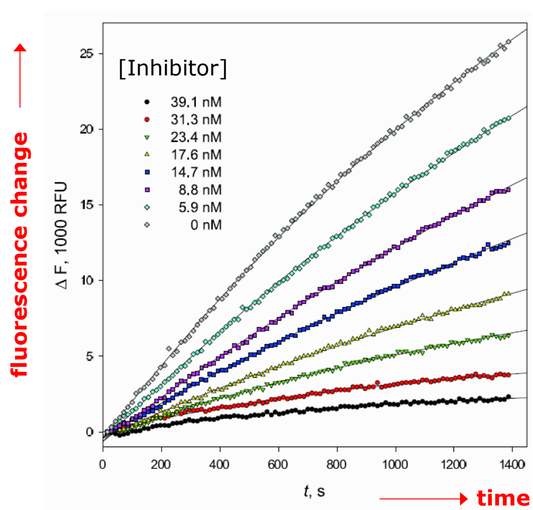
$$E + S \xrightarrow{k_{sub}} E + P$$

Goal of the study:

Evaluate the relative influence of
binding affinity and chemical reactivity
on cellular (biological) potency of each drug.

Example experimental data: Neratinib

NERATINIB VS. EFGR T790M / L858R DOUBLE MUTANT



Algebraic method of data analysis: Assumptions

The "textbook" method (based on **algebraic** rate equations):

Copeland R. A. (2013) "Evaluation of Enzyme Inhibitors in Drug Discovery", 2nd Ed., Eq. (9.1)(9.2)

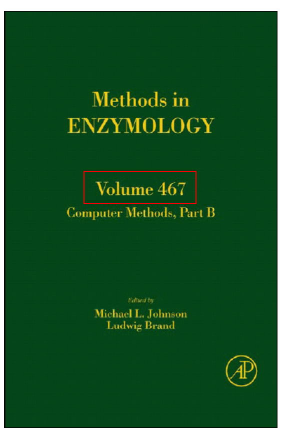
ASSUMPTIONS:

1. Control progress curve ([I] = 0) must be **strictly linear**
   - Negligibly small substrate depletion over the entire time course
2. Negligibly small **inhibitor depletion**
   - Inhibitor concentrations must be very much larger than K_i

Both of these assumptions are violated in our case.
The "textbook" method of kinetic analysis cannot be used.

An alternate approach: **Differential equation** formalism

"NUMERICAL" ENZYME KINETICS AND LIGAND BINDING



CHAPTER TEN

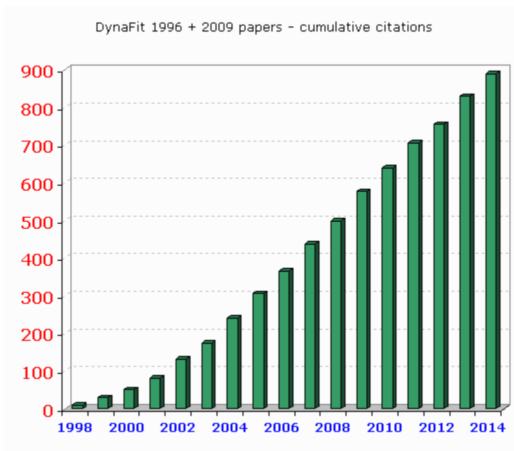
DYNAFIT—A SOFTWARE PACKAGE FOR ENZYMOLOGY

Petr Kuzmič

Kuzmic, P. (2009) *Meth. Enzymol.* **467**, 248-280

Kuzmic, P. (1996) *Anal. Biochem.* **237**, 260-273

DynaFit paper – Citation analysis



As of December 4, 2014:

- **892 citations**
- **50-60** citations per year
- Most frequently cited in:

Biochemistry	(39%)
J. Biol. Chem.	(23%)
J. Am. Chem. Soc.	(9%)
J. Mol. Biol.	(5%)
P.N.A.S.	(4%)
J. Org. Chem.	(4%)
...	

A "Kinetic Compiler"

HOW DYNAFIT PROCESSES YOUR BIOCHEMICAL EQUATIONS

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E.S \xrightarrow{k_3} E + P$$

Input (plain text file):

```
E + S ---> ES   :  k1

ES ---> E + S   :  k2

ES ---> E + P   :  k3
```

Rate terms:

$k_1 \times [E] \times [S]$

$k_2 \times [ES]$

$k_3 \times [ES]$

Rate equations:

$$d[\boldsymbol{E}] / dt = - k_1 \times [E] \times [S] + k_2 \times [ES] + k_3 \times [ES]$$

$$d[\boldsymbol{ES}] / dt = + k_1 \times [E] \times [S] - k_2 \times [ES] - k_3 \times [ES]$$

Similarly for other species...

System of Simple, Simultaneous Equations

HOW DYNAFIT PROCESSES YOUR BIOCHEMICAL EQUATIONS

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E.S \xrightarrow{k_3} E + P$$

"The LEGO method" of deriving rate equations

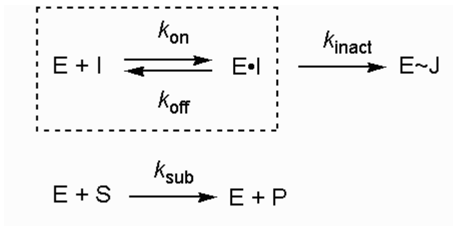
Input (plain text file):	Rate terms:	Rate equations:
`E + S ---> ES : k1`	$k_1 \times [E] \times [S]$	
`ES ---> E + S : k2`	$k_2 \times [ES]$	
`ES ---> E + P : k3`	$k_3 \times [ES]$	

DynaFit can analyze many types of experiments

MASS ACTION LAW AND **MASS CONSERVATION LAW** IS APPLIED TO DERIVE DIFFERENT MODELS

EXPERIMENT	DYNAFIT DERIVES A SYSTEM OF ...
Reaction progress	First-order ordinary differential equations
Initial rates	Nonlinear algebraic equations
Equilibrium binding	Nonlinear algebraic equations

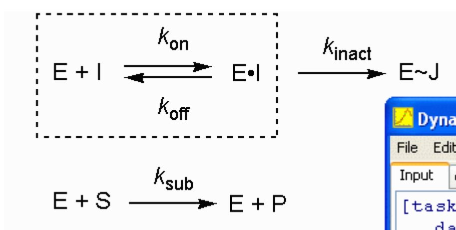
The differential equation model of covalent inhibition



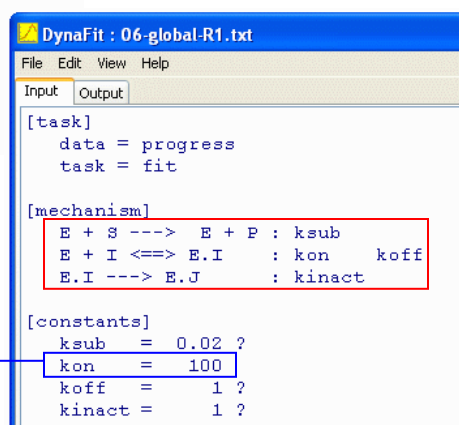
$$
\begin{aligned}
d[E]/dt &= - k_{sub}[E][S] + k_{sub}[E][S] - k_{on}[E][I] + k_{off}[E.I] \\
d[S]/dt &= - k_{sub}[E][S] \\
d[P]/dt &= + k_{sub}[E][S] \\
d[I]/dt &= - k_{on}[E][I] + k_{off}[E.I] \\
d[E.I]/dt &= + k_{on}[E][I] - k_{off}[E.I] - k_{inact}[E.I] \\
d[E.J]/dt &= + k_{inact}[E.I]
\end{aligned}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

This model is "integrated numerically".

Model of covalent inhibition in DynaFit



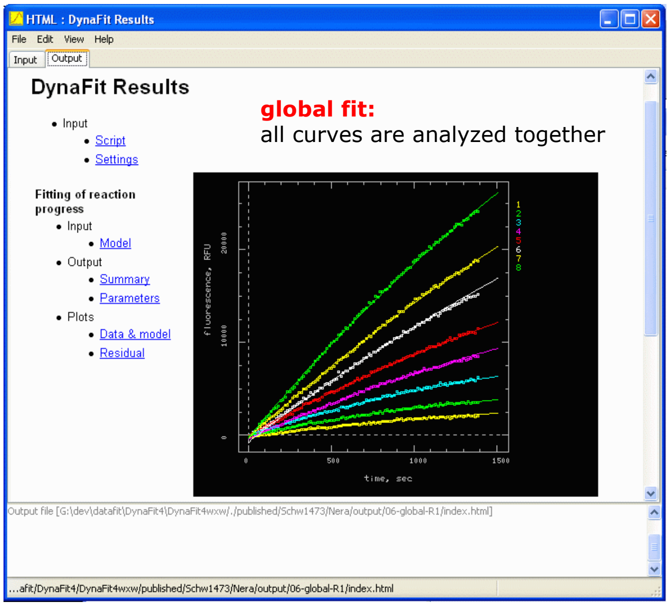
DynaFit input "script":



fixed constant:

"rapid-equilibrium approximation"

Covalent inhibition in DynaFit: Data / model overlay



Covalent inhibition in DynaFit: Model parameters

DynaFit output window:

Optimized Parameters

No.	Par#Set	Initial	Final	Std. Error	CV (%)
#1	ksub	0.02	**0.0141339**	0.000414818	2.93
#2	koff	1	**0.341161**	0.0125877	3.69
#3	kinact	1	**0.000862683**	5.67528e-005	6.58

How do we get **K_i** out of this?

- Recall that k_{on} was arbitrarily fixed at **100** **µM**-1s-1 ("rapid equilibrium")

K_i = k_{off}/k_{on} = 0.341 / **100** = 0.00341 µM = **3.4 nM**

K_i and k_{inact} as distinct determinants of cellular potency

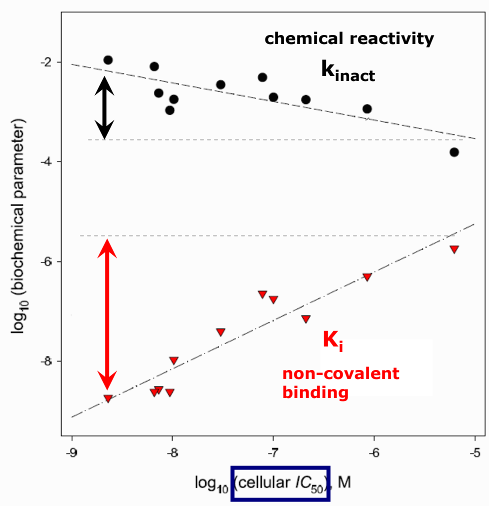


Fig. S 10: Correlation of covalent inhibitor kinetic constants toward EGFR-L858R/T790M with cellular potency (inhibition of EGFR-L858R/T790M autophosphorylation in H1975 tumor cells)

CORRELATION ANALYSIS:

Non-covalent initial binding affinity (**R^2 ~ 0.9**) correlates more strongly with **cellular potency**, compared to chemical reactivity (**R^2 ~ 0.5**).

Schwartz, Kuzmic, *et al.* (2014) Fig S10

K_i is a major determinant of cellular potency: Panel of 154

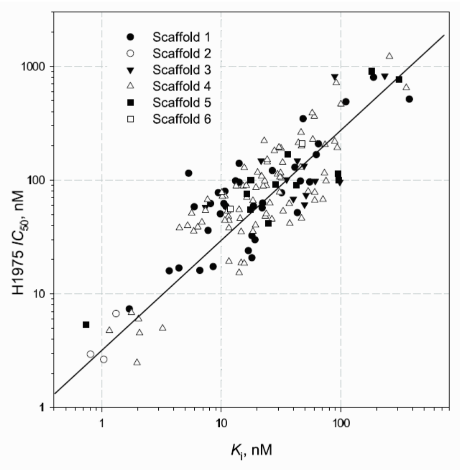


Fig. S 11: Correlation of dissociation constants of the initial non-covalent enzyme/inhibitor complex, as measured by the K_i values for EGFR-L858R/T790M double mutant, with cellular potency (inhibition of EGFR-L858R/T790M autophosphorylation in H1975 tumor cells) for 154 compounds spanning six structural classes.

Non-covalent K_i
vs.
Cellular IC_{50}

strong correlation for a larger panel

Schwartz, Kuzmic, *et al.* (2014) Fig S11

Overall conclusions, up to this point

Non-covalent initial binding
appears more important
than **chemical reactivity**
for the cellular potency
of this particular panel of
11 covalent anticancer drugs.

PNAS

Covalent EGFR inhibitor analysis reveals importance of reversible interactions to potency and mechanisms of drug resistance

Phillip A. Schwartz[a], Petr Kuzmic[b], James Solowiej[a], Simon Bergqvist[a], Ben Bolanos[c], Chau Almaden[a], Asako Nagata[c], Kevin Ryan[c], Junli Feng[c], Deepak Dalvie[d], John C. Kath[c], Meirong Xu[a], Revati Wani[a], and Brion William Murray[a,1]

[a]Oncology Research Unit, [c]Worldwide Medicinal Chemistry, and [d]Pharmacokinetics and Drug Metabolism, Pfizer Worldwide Research and Development, La Jolla, Pfizer Inc., San Diego, CA 92121; and [b]Research and Development, BioKin Ltd., Watertown, MA 02472

Edited* by Napoleone Ferrara, University of California, San Diego, La Jolla, CA, and approved November 25, 2013 (received for review July 23, 2013)

Proc. Natl. Acad. Sci. USA. **111**, 173-178 (2014).

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

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Confidence intervals for “on” / “off” rate constants

- We cannot determine “on” and “off” constants from currently available data.
- But we 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

Likelihood profile method: Computational algorithm

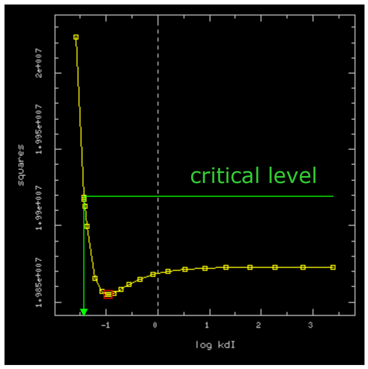
1. Perform nonlinear least-squares fit with the full set of model parameters.
2. Progressively **increase** a parameter of interest, **P**, away from its best-fit value. From now on keep **P** *fixed* in the fitting model.
3. At each step optimize the *remaining* model parameters.
4. Continue stepping with **P** until the sum of squares reaches a critical level.
5. This critical increase marks the **upper** end of the confidence interval for **P**.
6. Go back to step #2 and progressively **decrease** P, to find the **lower** end of the confidence interval.

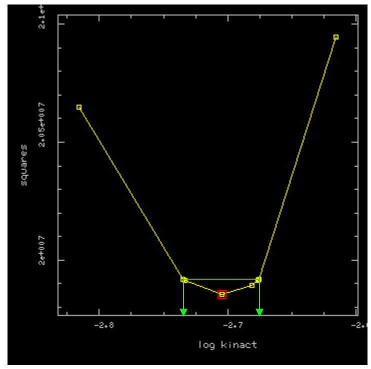
Watts, D.G. (1994)
"Parameter estimates from nonlinear models"
Methods in Enzymology, vol. **240**, pp. 23-36

Likelihood profile method: Example

Afatinib, replicate #1

sum of squares





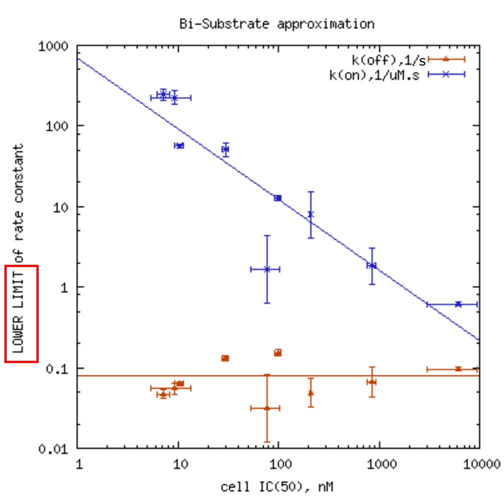
log (k_{off})

log (k_{inact})

lower end of confidence interval

lower and upper end of C.I.

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.

Lower limits vs. "true" values of rate constants

- We ***assumed*** that the lower limits for k_{on} and k_{off} are relevant proxies for "true" values.
- One way to validate this is via **Monte-Carlo** simulations:

1. Simulate many **artificial** data sets where the "true" value is known.
2. Fit each synthetic data set and determine confidence intervals.
3. Compare "true" (i.e. simulated) values with lower limits.

- Preliminary Monte-Carlo results confirm our assumptions.
- Extensive computations are currently ongoing.
- Publication is planned for early 2015.

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

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

- **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.

“Residence time” hypothesis of drug efficacy

SEMINAL PAPERS:

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

EXAMPLE SYSTEMS:

- work from Peter Tonge’s lab (SUNY Stony Brook)

ILLUMINATING DISCUSSION:

- Dahl & Akerud (2013) *Drug Disc. Today* **18**, 697-707

“Taking **pharmacokinetics** into consideration **limits the usability** of drug–target residence time as a predictor of the duration of effect for a drug *in vivo*.”

Summary and conclusions: Biochemical vs. cellular potency

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”).

CAVEAT: We only looked at **11** inhibitors of a **single** enzyme. *Additional work is needed to confirm our findings.*

Acknowledgments

- Brion Murray
- Philip Schwartz*
- Jim Solowiej

Pfizer Oncology
La Jolla, CA

* Currently Takeda Pharma
San Diego, CA

This presentation is available for download at **www.biokin.com**

biochemical
kinetics

SUPPLEMENTARY SLIDES

CHECK UNDERLYING ASSUMPTIONS:
BIMOLECULAR ASSOCIATION RATE

Differential equation method: Example – Afatinib: Parameters

DYNAFIT-GENERATED OUTPUT

recall:
we
assumed
this value

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

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

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}

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

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

ASSUMED	DETERMINED FROM DATA			
$\mathbf{k_{aI}}$, $\mu M^{-1}s^{-1}$	$\mathbf{k_{inact}}$, s^{-1}	$\mathbf{k_{dI}}$, s^{-1}	$\mathbf{K_i}$, nM	$\mathbf{k_{inact}/K_i}$, $\mu M^{-1}s^{-1}$
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, $\mathbf{K_i}$, **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 $\mathbf{k_{inact}}$, $\mathbf{K_i}$, and $\mathbf{k_{inact}/K_i}$.

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

CHECK UNDERLYING ASSUMPTIONS:
SUBSTRATE MECHANISM

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Substrate mechanism – "Hit and Run"

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

$$E + S \xrightarrow{k_{sub}} E + P$$

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

- 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)

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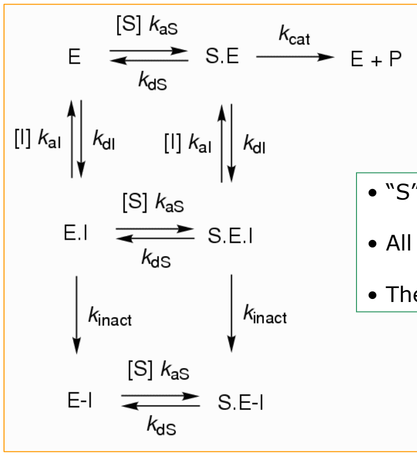
Substrate mechanism – Michaelis-Menten

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**

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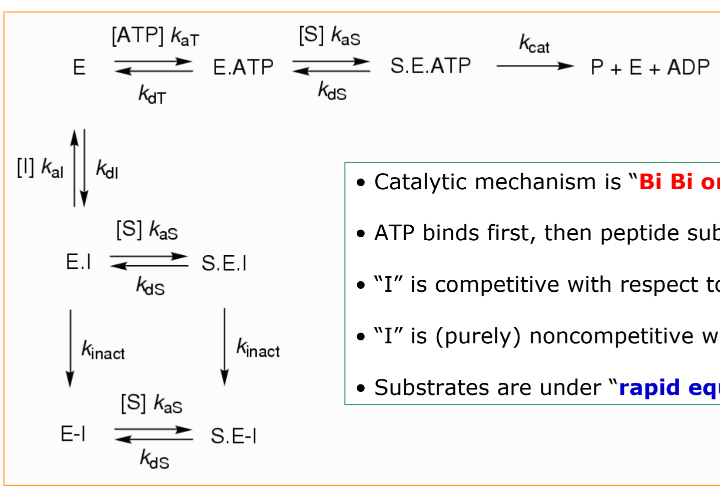
Substrate mechanism – Bi-Substrate



- 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**"

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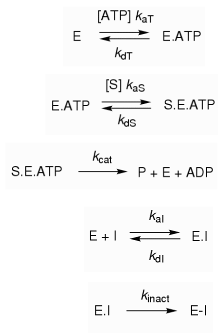
Substrate mechanism – “Bi-Substrate”: DynaFit notation

MECHANISM:



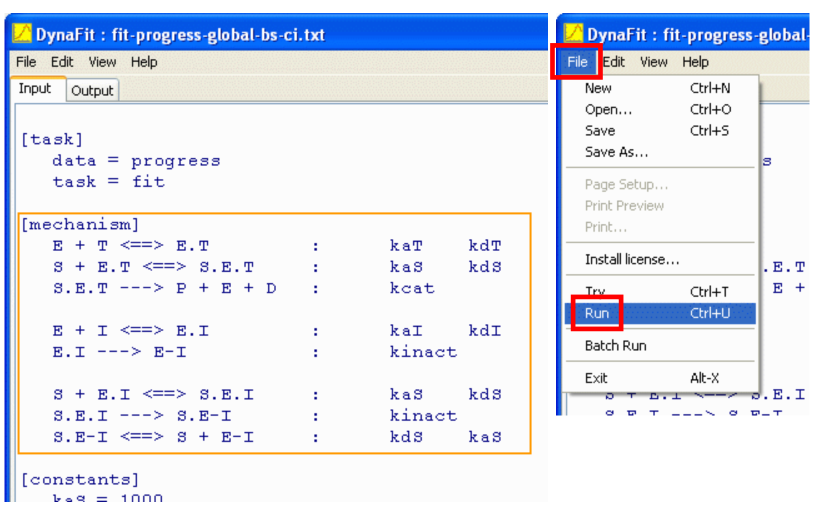
DYNAFIT INPUT:

```
[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 $\mathbf{k_{aI}}$, $\mu M^{-1}s^{-1}$	$\mathbf{k_{dI}}$, s^{-1}	$\mathbf{k_{inact}}$, s^{-1}	$\mathbf{k_{dI}/k_{aI}}$ $\mathbf{K_i}$, 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

$\mathbf{[ATP]/K_{M,ATP}}$ = **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.

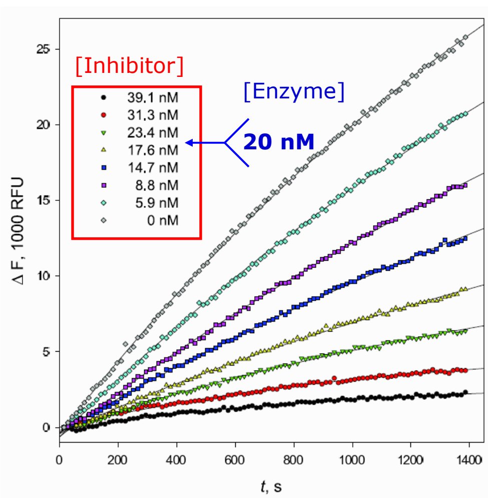
3. The initial binding affinity ($\mathbf{K_i}$) needs to be **corrected for ATP** competition in the case of “Hit and Run” and “Michaelis-Menten” mechanisms:

   - 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.

THEORETICAL ASSUMPTIONS VIOLATED:
CLASSIC ALGEBRAIC METHOD

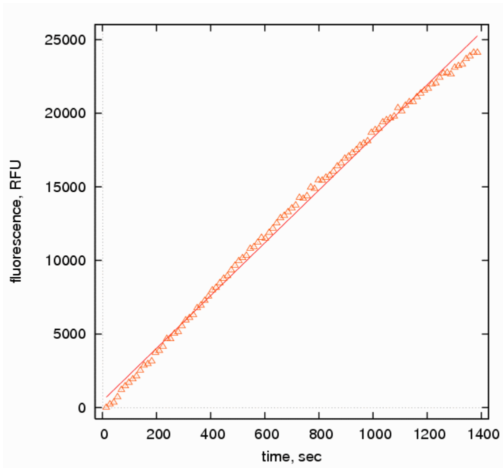
Check concentrations: "Tight binding" or not?



The assumption that [Inhibitor] >> [Enzyme] clearly does not hold.

We have **"tight binding"**, making it impossible to utilize the classic algebraic method.

Check linearity of control progress curve ([Inhibitor] = 0)



This "slight" nonlinearity has a massive impact, making it impossible to utilize the classic algebraic method:

REFERENCE:

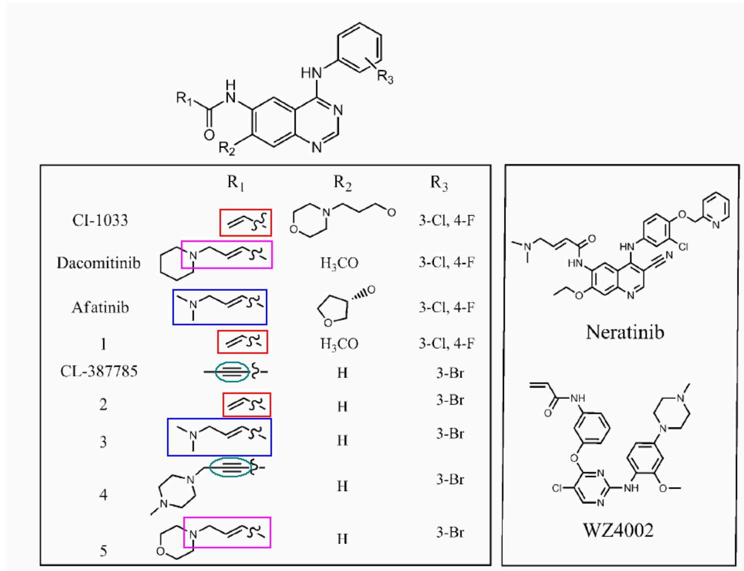
Kuzmic *et al.* (2015)

"An algebraic model for the kinetics of covalent enzyme inhibition at low substrate concentrations"

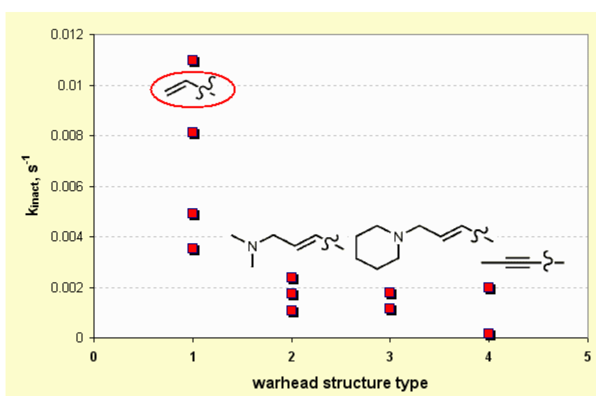
Anal. Biochem., in press
Manuscript No. **ABIO-14-632**

ACRYLAMIDE WARHEAD:
STRUCTURE VARIATION VS. k_{inact}

Caveat: Small number of warhead structures in the test panel



Warhead structure type vs. inactivation reactivity



1. large variation of reactivity for a single structure type (CH_2=CH-)
2. small variation of reactivity across multiple structure types