Application of Enzyme Kinetics
in the Development of Therapeutic Inhibitors

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BioKin, Ltd.

Guangzhou
December 2024
Expanded

Topics

1. Theory
2. Software
3. Examples
4. Discussion

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Topics

1. Theory
2. Software
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Three main tasks of enzyme inhibition kinetics

1. Determine the enzyme inhibition **mechanism**.
2. Determine the enzyme/inhibitor **binding affinity** (K_d).
3. Determine the **rate constants** for association and dissociation.

OUR GUIDING PRINCIPLE:

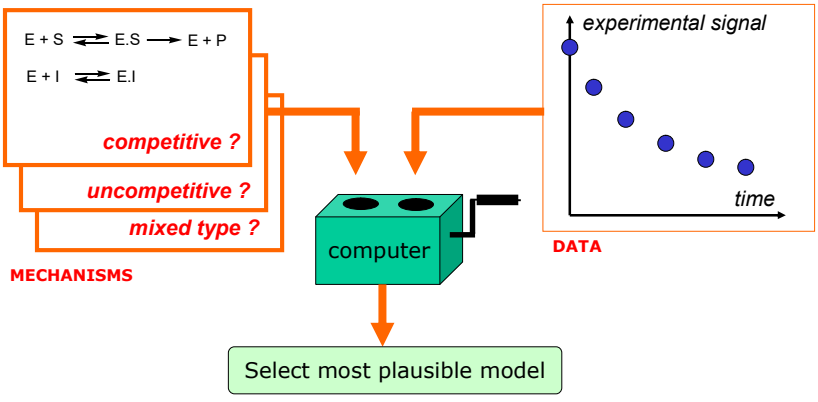
Hunches and intuitive impressions are essential for getting the work started, but it is only through the quality of the numbers at the end that the truth can be told.

*Lewis Thomas**
Memorial Sloan-Kettering Cancer Center

*L. Thomas, "Biostatistics in Medicine," *Science* **198**:675, 1977.

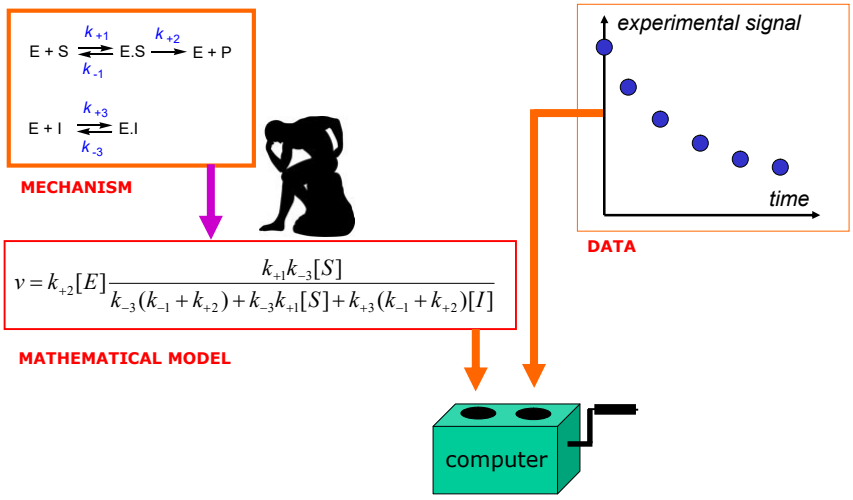
The first task of enzyme inhibition kinetics

SELECT AMONG MULTIPLE POSSIBLE INHIBITION **MECHANISMS**



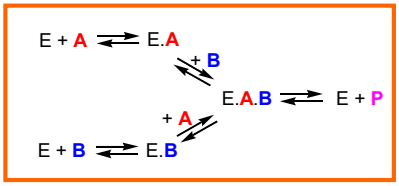
From mechanistic to mathematical models

DERIVE A MATHEMATICAL MODEL FROM BIOCHEMICAL/BIOPHYSICAL IDEAS



Problem: Simple mechanisms ...

MERELY FIVE REACTIONS ...



"RANDOM BI-UNI" MECHANISM

- 2 reactants (A, B)
- 1 product (P)

- 5 reversible reactions
- 10 rate constant

... lead to highly complex algebraic models

Segel, I. (1975) *Enzyme Kinetics*. John Wiley, New York, p. 646.

$$\frac{v}{[E]_t} = \frac{K_1[A][B] + K_2[A]^2[B] + K_3[A][B]^2 - K_4[P] - K_5[A][P] - K_6[B][P]}{K_7 + K_8[A] + K_9[B] + K_{10}[A][B] + K_{11}[A]^2 + K_{12}[B]^2 + K_{13}[A]^2[B]}$$

$$+ K_{14}[A][B]^2 + K_{15}[P] + K_{16}[A][P] + K_{17}[B][P] + K_{18}[A][B][P] \qquad (IX\text{-}181)$$

($k_{-5} = 0$)

where K_1 through K_{18} represent combinations of rate constants:

$K_1 = k_1k_{-2}k_3k_5 + k_{-1}k_2k_4k_5$, $K_2 = k_1k_3k_4k_5$, $K_3 = k_2k_3k_4k_5$,

$K_4 = k_{-1}k_{-2}k_{-3}k_{-5} + k_{-1}k_{-2}k_{-4}k_{-5}$, $K_5 = k_{-1}k_{-3}k_4k_{-5}$,

$K_6 = k_{-2}k_3k_{-4}k_{-5}$, $K_7 = k_{-1}k_{-2}k_{-3} + k_{-1}k_{-2}k_{-4} + k_{-1}k_{-2}k_5$,

$K_8 = k_1k_{-2}k_{-3} + k_1k_{-2}k_{-4} + k_1k_{-2}k_5 + k_{-1}k_{-3}k_4 + k_{-1}k_4k_5$,

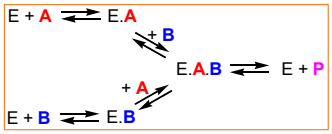
$K_9 = k_{-1}k_2k_{-3} + k_{-1}k_2k_{-4} + k_{-1}k_2k_5 + k_{-2}k_3k_{-4} + k_{-2}k_3k_5$,

$K_{10} = k_1k_{-2}k_3 + k_{-1}k_2k_4 + k_1k_3k_{-4} + k_2k_{-3}k_4 + k_3k_4k_5$,

$K_{11} = k_1k_{-3}k_4 + k_1k_4k_5$, $K_{12} = k_2k_3k_{-4} + k_2k_3k_5$, $K_{13} = k_1k_3k_4$,

$K_{14} = k_2k_3k_4$, $K_{15} = k_{-1}k_{-2}k_{-5} + k_{-1}k_{-4}k_{-5} + k_{-2}k_{-3}k_{-5}$,

$K_{16} = k_{-1}k_4k_{-5} + k_{-3}k_4k_{-5}$, $K_{17} = k_{-2}k_3k_{-5} + k_3k_{-4}k_{-5}$, $K_{18} = k_3k_4k_{-5}$



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New approach: Numerical Kinetics

NO MORE ALGEBRA! LET THE **COMPUTER** DEAL WITH IT

Theoretical foundations: *Mass Action Law*

RATE IS PROPORTIONAL TO CONCENTRATION(S)

MONOMOLECULAR REACTIONS

A ⟶ products

rate is proportional to **[A]**

$-\,d[A]/dt = k[A]$

BIMOLECULAR REACTIONS

A + B ⟶ products

rate is proportional to **[A] × [B]**

$-\,d[A]/dt = -\,d[B]/dt = k[A] \times [B]$

Theoretical foundations: *Mass Conservation Law*

PRODUCTS ARE FORMED WITH THE SAME RATE AS REACTANTS DISAPPEAR

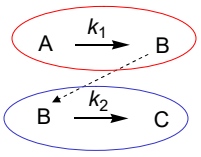
EXAMPLE

A ⟶ P + Q

$-\,d[\mathbf{A}]/dt = +d[\mathbf{P}]/dt = +d[\mathbf{Q}]/dt$

COMPOSITION RULE ADDITIVITY OF TERMS FROM SEPARATE REACTIONS

mechanism:

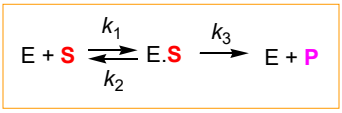


$d[B]/dt = +k_1[A] - k_2[B]$

Computer-assisted derivation of mathematical models

TIME-COURSE OF ENZYME REACTIONS: SYSTEMS OF **DIFFERENTIAL EQUATIONS**

Step 1



mental
model

```
E + S <==> E.S  :  k1  k2
E.S ---> E + P  :  k3
```

symbolic
computer
encoding

Computer-assisted derivation of mathematical models

TIME-COURSE OF ENZYME REACTIONS: SYSTEMS OF **DIFFERENTIAL EQUATIONS**

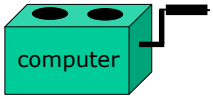
Step 2

DynaFit : der-001.txt

File Edit View Help

Input Output

```
[task]
   task = derive
   data = progress
[mechanism]
   E + S <==> E.S  :  k1  k2
   E.S ---> E + P  :  k3
[output]
   directory ^/ODE-michaelis-menten
[end]
```



HTML : Model

File Edit View Help

Input Output

ODE system

$d[E]/dt = -k_1[E][S] + k_2[E.S] + k_3[E.S]$

$d[S]/dt = -k_1[E][S] + k_2[E.S]$

$d[E.S]/dt = +k_1[E][S] - k_2[E.S] - k_3[E.S]$

$d[P]/dt = +k_3[E.S]$

Jacobian matrix

	E	S	E.S	P
E	$-k_1[S]$	$-k_1[E]$	$+k_2+k_3$	0
S	$-k_1[S]$	$-k_1[E]$	$+k_2$	0
E.S	$+k_1[S]$	$+k_1[E]$	$-k_2-k_3$	0
P	0	0	$+k_3$	0

symbolic
computer
encoding

internal
computer
representation

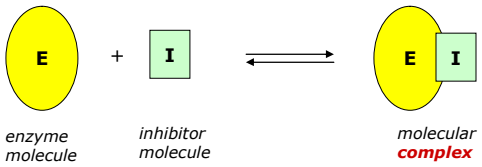
What is enzyme inhibition on the molecular level

COMBINATION OF TWO MOLECULES TO FORM AN **ENZYME-INHIBITOR COMPLEX**

"Drugs produce their inhibitory action by combining with the enzyme [molecules]."

"One molecule of drug will inhibit the activity of one [molecule] of enzyme."

Easson, L. H. & Stedman, E. (1936) *Proc. Roy. Soc. B* **121**, 142-151.



What is the inhibition constant (K_i)

DISSOCIATION EQUILIBRIUM CONSTANT OF THE ENZYME-INHIBITOR COMPLEX

$$E + I \rightleftharpoons E{\cdot}I \qquad K_i = \frac{[E]_{eq}\,[I]_{eq}}{[E \cdot I]_{eq}}$$

- *low* K_i ("dissociation") means *high* binding activity
- dimension = *concentration* (moles/liter, **M**)
- "good" inhibitors have K_i's around 10^{-9} moles/liter or better (**nano**molar)

10^{-3}	*mili-*	**m**M
10^{-6}	*micro-*	**µ**M
10^{-9}	*nano-*	**n**M
10^{-12}	*pico-*	**p**M
10^{-15}	*femto-*	**f**M
10^{-18}	*atto-*	

"better" inhibitor

Measures of inhibitor binding affinity

INTRINSIC MEASURE OF POTENCY:

$\Delta G = -RT \log K_i$

DEPENDENCE ON EXPERIMENTAL CONDITIONS	Depends on [S]	Depends on [E]	*Example:* Competitive inhibitor
1. Inhibition constant	**NO**	**NO**	K_i
2. Apparent K_i	**YES**	**NO**	$K_i^* = K_i (1 + [S]/K_M)$
3. IC_{50}	**YES**	**YES**	$IC_{50} = K_i (1 + [S]/K_M) + [E]/2$

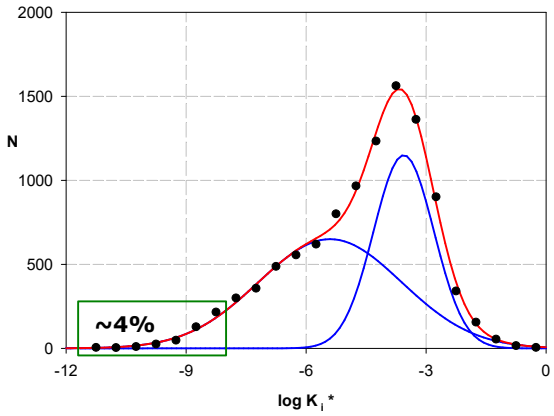
"CLASSICAL" INHIBITORS: $[E] \ll K_i$: $IC_{50} \approx K_i^*$

"TIGHT BINDING" INHIBITORS: $[E] \approx K_i$: $IC_{50} \neq K_i^*$

Tight binding inhibitors : $[E] \approx K_i$

HOW PREVALENT IS "TIGHT BINDING"?

A typical data set: ~ 10,000 **compounds**
Completely inactive: ~ 1,100 ... NOT SHOWN
Tight binding: ~ 400



Data courtesy of Celera Genomics

What is the difference between K_i and IC_{50} ?

IC_{50} **DEPENDS ON ENZYME** CONCENTRATION AND IS **ALWAYS HIGHER** THAN THE K_i

$$IC_{50} = \frac{[E]_0}{2} + K_i^{(app)}$$

$$K_i^{(app)} = K_i(1 + [S]/K_M) \quad \textbf{competitive}$$

$$K_i^{(app)} = K_i(1 + K_M/[S]) \quad \textbf{uncompetitive}$$

$$K_i^{(app)} = K_i \quad \textbf{noncompetitive}$$

$$K_i^{(app)} = \frac{[S] + K_M}{[S]/\alpha K_i + K_M/K_i} \quad \textbf{mixed-type}$$

Cha, S. (1975) "Tight binding inhibitors. I. Kinetic behavior" *Biochem. Pharmacol.* **24**, 2177-2185.

Implications for drug discovery: "Hitting the IC_{50} wall"

NO MATTER HOW TIGHTLY THE INHIBITOR BINDS, THE **IC_{50} CAN NEVER GET LOWER** THAN $[E]_0/2$

Assume: $K_i^{(app)} = K_i\,(1 + [S]/K_M)$

- **competitive**
- **[E] = 5 nM**
- **$[S]_0 = K_M$**

K_i, nM	IC_{50}, nM
1,000	2,002.5
100	202.5
10	22.5
1	4.5
0.1	2.6
0.01	2.52
0.001	2.502

- **competitive**
- **[E] = 60 nM**
- **$[S]_0 = K_M$**

K_i, nM	IC_{50}, nM
1,000	2,030
100	230
10	50
1	**32**
0.1	**30**.2
0.01	**30**.02
0.001	**30**.002

The IC_{50} wall.

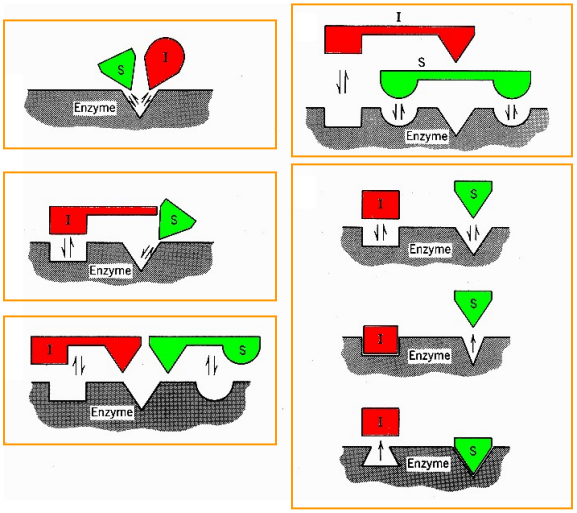
Enzyme inhibition "modality"

THE FOUR MAJOR TYPES OF ENZYME INHIBITION

Mode	Explanation
competitive	binding of substrate and inhibitor is mutually exclusive
noncompetitive	inhibitor binds to a non-substrate site and the binding affinity of substrate is **unaffected**
mixed-type	inhibitor binds to a non-substrate site and the binding affinity of substrate is **affected**
uncompetitive	inhibitor binds only to the enzyme-substrate complex (applicable only to multi-substrate enzymes)

Competitive inhibition kinetics vs. structure

"COMPETITIVE" INHIBITION KINETICS DOES **NOT ALWAYS** MEAN STRUCTURAL COMPETITION !



S = substrate

I = inhibitor

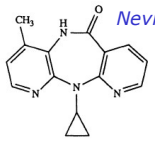
Segel, I. (1975) *Enzyme Kinetics*, John Wiley, New York, p. 102

Noncompetitive inhibition kinetics vs. structure

"NON-COMPETITIVE" INHIBITION KINETICS **DOES** MEAN MULTIPLE BINDING SITES

HIV-1 Reverse Transcriptase

Nevirapine



Kinetics

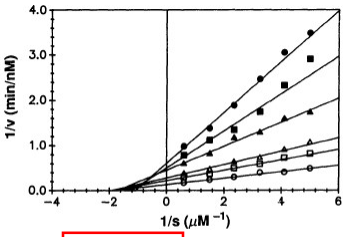


Fig. 2. Noncompetitive inhibition was determined from a double reciprocol plot for inhibition of RT by BI-RG-587 with dGTP as variable

Merluzzi et al. (1990) *Science* **250**, 1411

Structure

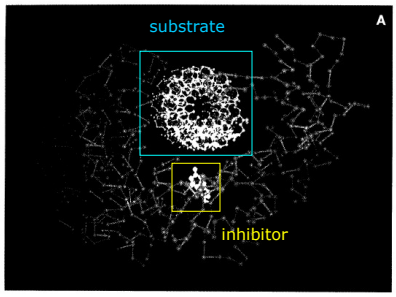


Fig. 6. The position of the inhibitor, Nevirapine (*2*), bound to the p66 pol active site near to the catalytic site and the expected DNA primer

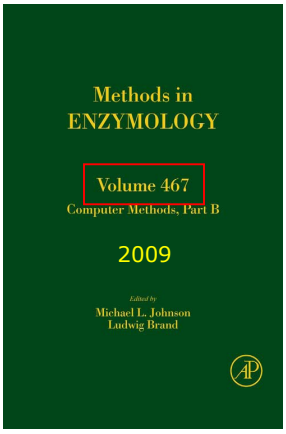
Kohlstaedt et al. (1992) *Science* **256**, 1783

Topics

1. Theory
2. Software
3. Examples
4. Discussion

Specialized numerical software: *DynaFit*

APPROXIMATELY **1,800 JOURNAL ARTICLES** USED AND CITED DYNAFIT (1996 – 2024)



CHAPTER TEN

DYNAFIT—A SOFTWARE PACKAGE FOR ENZYMOLOGY

Petr Kuzmič

DOWNLOAD http://www.biokin.com/dynafit

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

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

DynaFit can analyze many types of experiments

MASS ACTION LAW AND MASS CONSERVATION LAW IS APPLIED IN THE SAME WAY

chemistry
biophysics
pharmacology
enzymology

EXPERIMENT	DYNAFIT DERIVES A SYSTEM OF …
Kinetics (time-course)	Ordinary differential equations (ODE)
Equilibrium binding	Nonlinear algebraic equations
Initial reaction rates	Nonlinear algebraic equations

DynaFit compared with similar software packages

DYNAFIT IS **THE ONLY SOFTWARE** IN THE WORLD THAT PASSES THE **N.I.S.T. NLREG TEST SUITE**

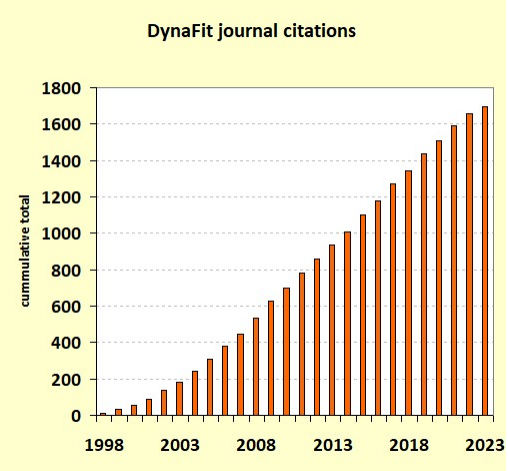
Software	*Author*	*Year*	*ODE solver algorithm*	*LSQ fitter algorithm*
KINSIM	Frieden	1983	Gear	--
FITSIM	Barshop	1986	Gear	Marquardt
DynaFit *v.* 1	Kuzmic	1996	LSODE	Marquardt
KinTek	Johnson	2009	(proprietary)	(proprietary)
DynaFit *v.* 4	Kuzmic	2017	LSODE	NL2SOL

Kuzmic, P. (2009) "DynaFit – A Software Package for Enzymology"
Methods in Enzymology **467**, 248-280

DOWNLOAD : **biokin.com**

DynaFit – Citation analysis (the first 25 years)

APPROXIMATELY **1,700 JOURNAL ARTICLES** USED AND CITED DYNAFIT (1998 – 2023)



- Most frequently cited in:

Biochemistry	(40%)
J. Biol. Chem.	(25%)
J. Am. Chem. Soc.	(10%)
J. Mol. Biol.	(5%)
P.N.A.S.	(5%)
...	
...	

Topics

1. Theory
2. Software
3. Example 1: Epidermal Growth Factor Receptor
4. Discussion

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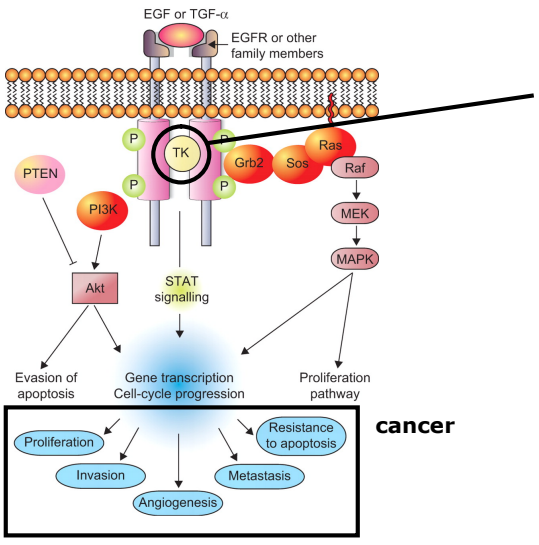
The target enzyme: Epidermal Growth Factor Receptor (EGFR)



tyrosine kinase activity

kinase inhibitors act as anticancer therapeutics

cancer

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

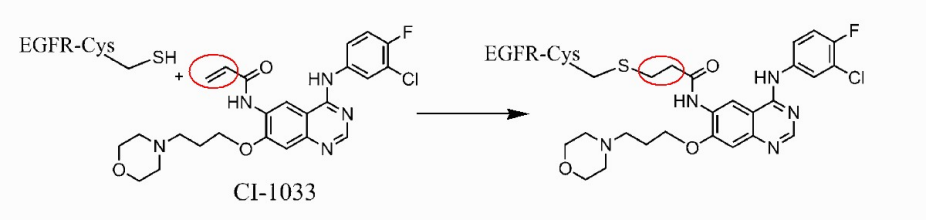
EGFR inhibition by covalent drugs

Schwartz, P.; Kuzmic, P. *et al*. (2014)

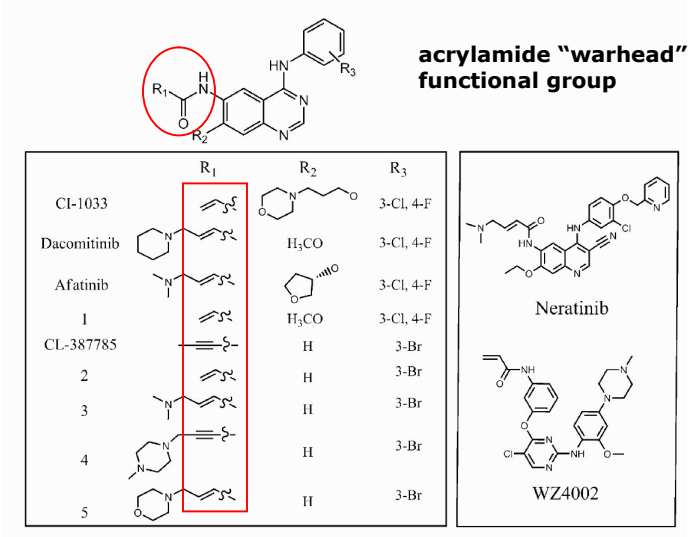
"Covalent EGFR inhibitor analysis reveals
importance of reversible interactions
to potency and mechanisms of drug resistance"

Proc. Natl. Acad. Sci. USA. **111**, 173-178. Issue 1, January 7

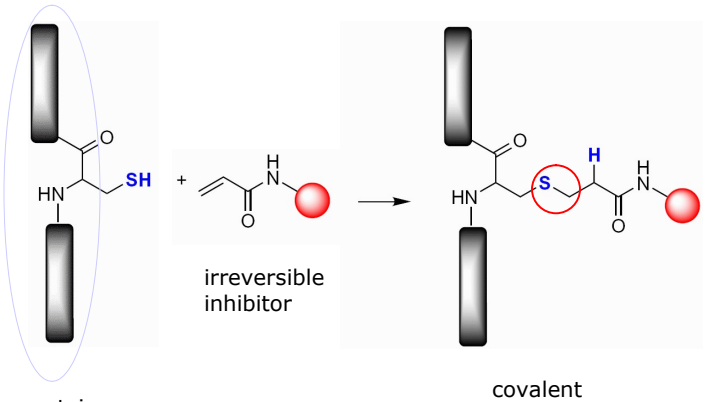
EXAMPLE:



EGFR kinase inhibitors in the test panel

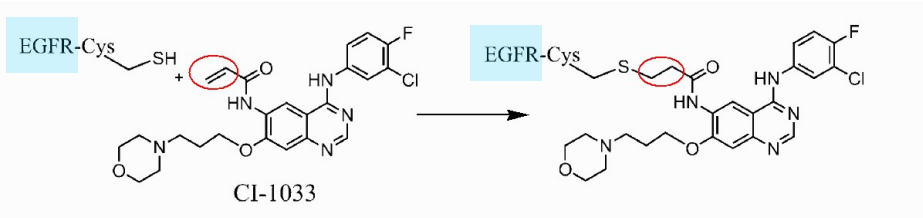


Covalent inhibitors of cancer-related enzymes: Mechanism



EGFR inhibition by covalent drugs: Example

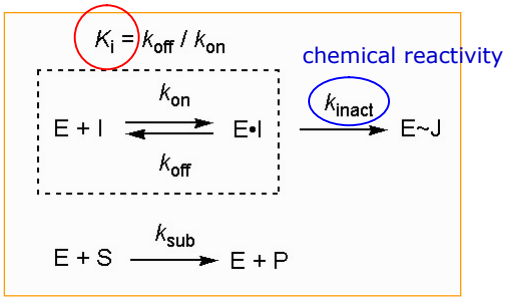
Michael addition of a cysteine –SH group



Canertinib (CI-1033): experimental cancer drug candidate

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

binding affinity

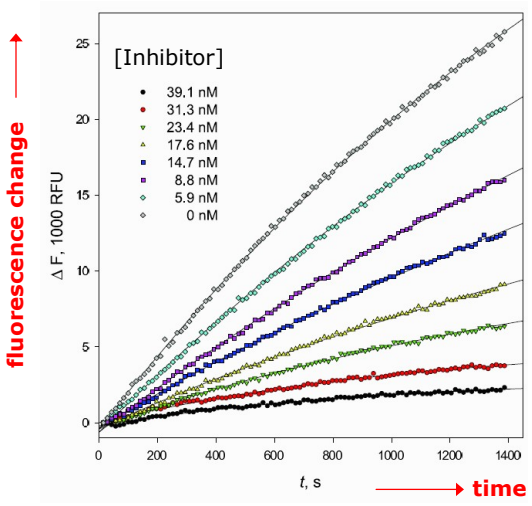


Goal of the study:

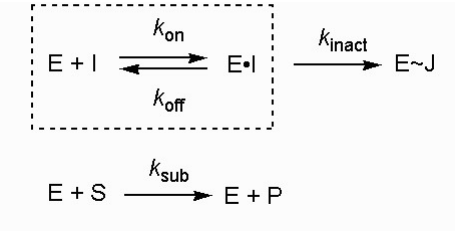
Evaluate the relative influence of
binding affinity and chemical reactivity
on cellular (biological) potency of each drug.

Example experimental data: Neratinib

NERATINIB VS. EFGR T790M / L858R DOUBLE MUTANT



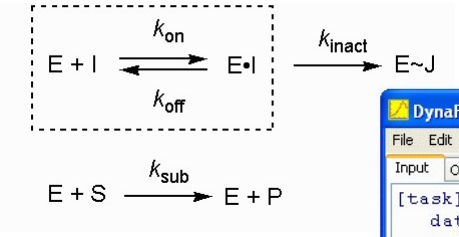
The differential equation model of covalent inhibition



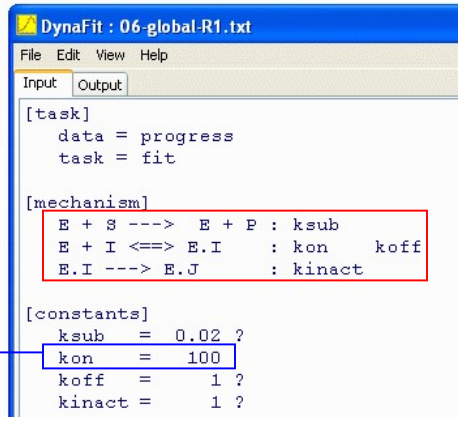
$$\frac{d[E]}{dt} = -k_{sub}[E][S] + k_{sub}[E][S] - k_{on}[E][I] + k_{off}[E.I]$$
$$\frac{d[S]}{dt} = -k_{sub}[E][S]$$
$$\frac{d[P]}{dt} = +k_{sub}[E][S]$$
$$\frac{d[I]}{dt} = -k_{on}[E][I] + k_{off}[E.I]$$
$$\frac{d[E.I]}{dt} = +k_{on}[E][I] - k_{off}[E.I] - k_{inact}[E.I]$$
$$\frac{d[E.J]}{dt} = +k_{inact}[E.I]$$

This model is "integrated numerically".

Model of covalent inhibition in DynaFit



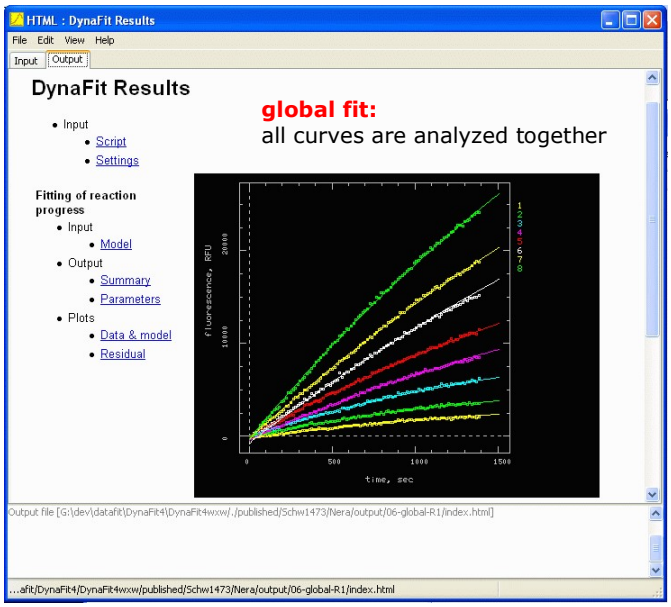
DynaFit input "script":



fixed constant:

"rapid-equilibrium approximation"

Covalent inhibition in DynaFit: Data / model overlay



Covalent inhibition in DynaFit: Model parameters

DynaFit output window:

Optimized Parameters

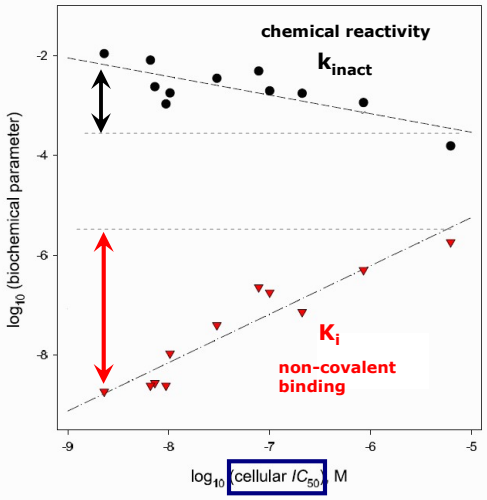
No.	Par#Set	Initial	Final	Std. Error	CV (%)
#1	ksub	0.02	**0.0141339**	0.000414818	2.93
#2	koff	1	**0.341161**	0.0125877	3.69
#3	kinact	1	**0.000862683**	5.67528e-005	6.58

How do we get K_i out of this?

- k_{on} was arbitrarily fixed at **100 μM^{-1}s^{-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



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

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)

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Topics

1. Theory
2. Software
3. Example 2: Innosine 5'-monophosphate dehydrogenase
4. Discussion

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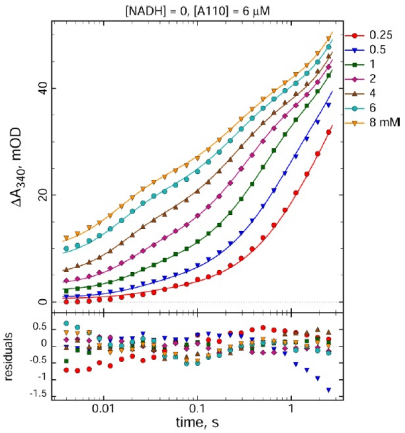
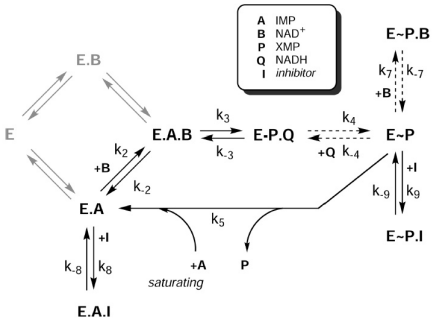
Transient kinetic model for *Bacillus anthracis* IMPDH

Wei, Y.; Kuzmic, P.; *et al.* (2016) *Biochemistry* **55**, 5279



Data



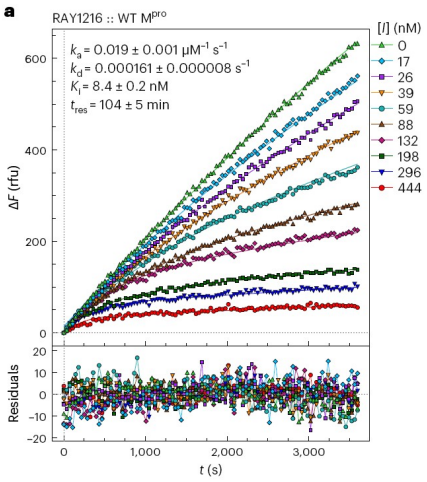
Mechanism
11 rate constants determined !

Topics

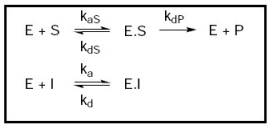
1. Theory
2. Software
3. Example 3: SARS-CoV-2 main protease / RAY1216
4. Discussion

RAY1216 (Leritrelvir) is a "slow, tight" inhibitor of M^{pro}

Chen, X; Huang, X.; Ma, Q.; Kuzmic, P., *et al.* (2024) *Nature Microbiology* **9**, 1075



Data



$$\frac{d[E]}{dt} = -k_{aS}[E][S] + k_{dS}[E.S] + k_{dP}[E.S] - k_a[E][I] + k_d[E.I] \quad (S6)$$

$$\frac{d[S]}{dt} = -k_{aS}[E][S] + k_{dS}[E.S] \quad (S7)$$

$$\frac{d[E.S]}{dt} = +k_{aS}[E][S] - k_{dS}[E.S] - k_{dP}[E.S] \quad (S8)$$

$$\frac{d[P]}{dt} = +k_{dP}[E.S] \quad (S9)$$

$$\frac{d[I]}{dt} = -k_a[E][I] + k_d[E.I] \quad (S10)$$

$$\frac{d[E.I]}{dt} = +k_a[E][I] - k_d[E.I] \quad (S11)$$

Model

as published; may need a slight revision

BioKin L t d.

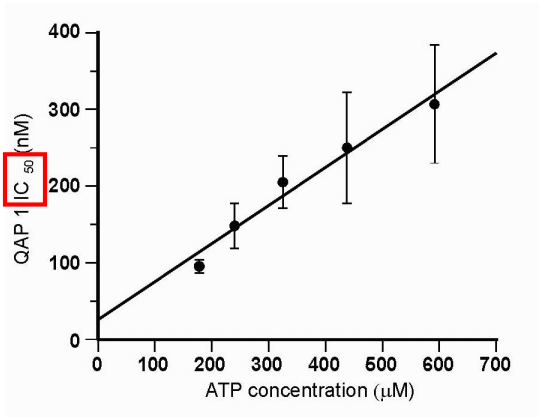
Topics

1. Theory
2. Software
3. Examples
4. Discussion

BioKin L t d.

Some people still use "IC_{50}" to determine inhibition modality

A PAPER FROM **NOVARTIS PHARMA A.G. (SWITZERLAND)**

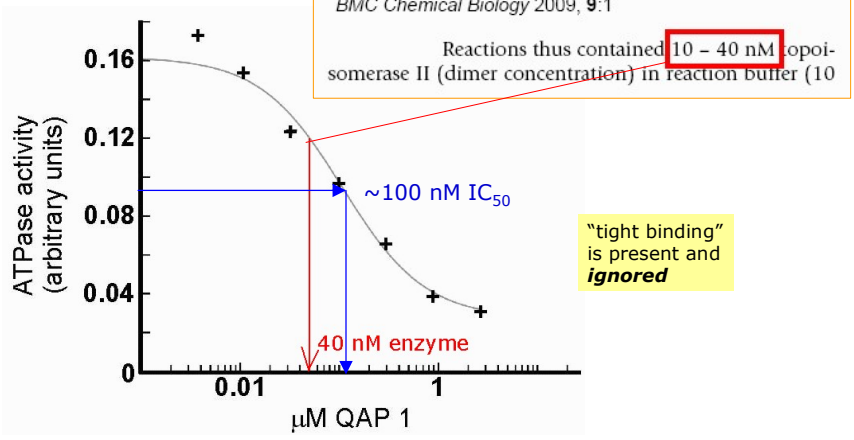


Catalytic inhibition of topoisomerase II by a novel rationally designed ATP-competitive purine analogue

Chene, P. *et al.* (2009) *BMC Chem. Biol.* **9**:1, doi:10.1186/1472-6769-9-1

Some people still use "IC_{50}" to determine inhibition modality

A PAPER FROM **NOVARTIS PHARMA A.G. (SWITZERLAND)**



Chene, P. *et al.* (2009) *BMC Chem. Biol.* **9**:1, doi:10.1186/1472-6769-9-1

Other people insist that "IC_{50}" is a perfectly sufficient method

A PAPER FROM **PFIZER INC. (U.S.A.)**

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

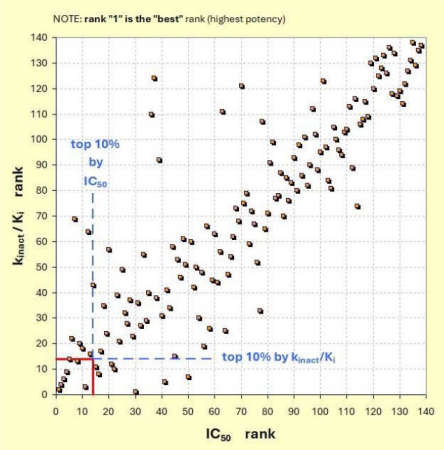
Atli Thorarensen [a,*], Paul Balbo [b], Mary E. Banker [c], Robert M. Czerwinski [b], Max Kuhn [d], Tristan S. Maurer [a], Jean-Baptiste Telliez [b], Fabien Vincent [c], Arthur J. Wittwer [b]

[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

Other people insist that "IC_{50}" is a perfectly sufficient method

A PAPER FROM **PFIZER INC. (U.S.A.)**

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



The "top 10%" rule by IC_{50} is only about 50% efficient: it misses 1/2 of true "10%" hits.

A personal opinion: True innovation in enzymology is lacking

"BIG PHARMA" APPEARS TO **ACTIVELY REFUSE TO INNOVATE** IN ENZYME KINETICS

https://www.science.org/doi/10.1126/science.adu7982
Parikh, S.S.; *et al.* (2024) *Science* **386**, 947
Published November 29, 2024

A new vision for American science

"[W]hat got America to this point will not get the country to where it needs to go. **A new vision is required** to respond to an evolving global science and technology ecosystem. [...] The country risks ceding discovery and development - and their economic rewards - to nations that have plans to act and greater will to invest."

Sudip S. Parikh
CEO of the American Association for the Advancement of Science
Marcia K. McNutt
President of the US National Academy of Sciences
Darío Gil
Chair of the National Science Board, Director of Research at I.B.M.

Summary and Conclusions

- The "old" enzymology, based on **algebra**, has been replaced by "new" enzymology, based on **numerical** methods.
- This innovation allows us to advance **drug research** in important ways:
    1. perform experiments under **arbitrary conditions**; and
    2. correctly handle **"slow", "tight"** enzyme inhibitors.
- The adoption of this new approach (cca. 2000) has been fairly slow. Traditional kinetic methods (cca. 1960-1980) persist in the literature.
- A **new generation** of enzymologists and drug discovery experts should receive better traning in this area.