Why IC_{50}'s Are Bad for You

And Other Surprises

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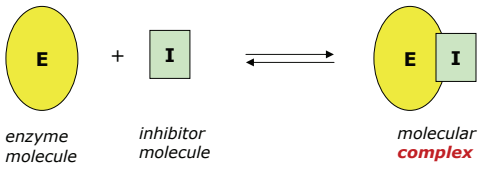
What is enzyme inhibition on the molecular level

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-*	

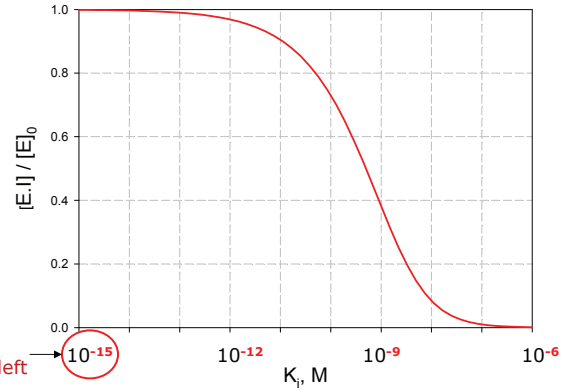
"better" inhibitor

A thought experiment: Effect of K_i on "fraction bound"

$$E + I \rightleftharpoons E{\cdot}I$$

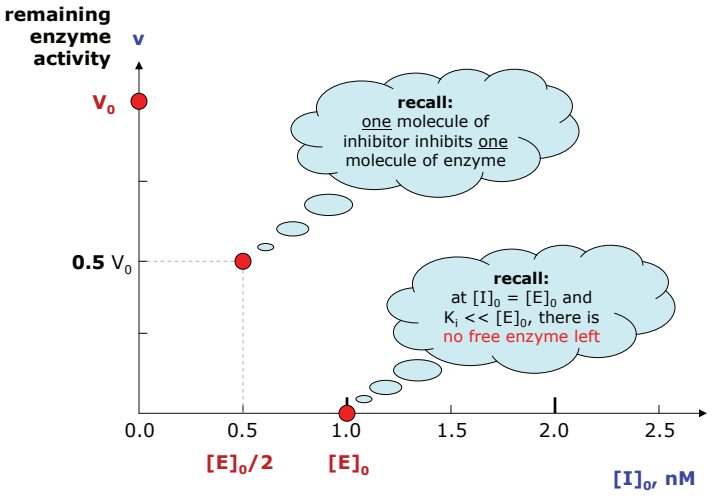
Assume *equal amounts:*

$[E]_0 = [I]_0 =$ 1 nM



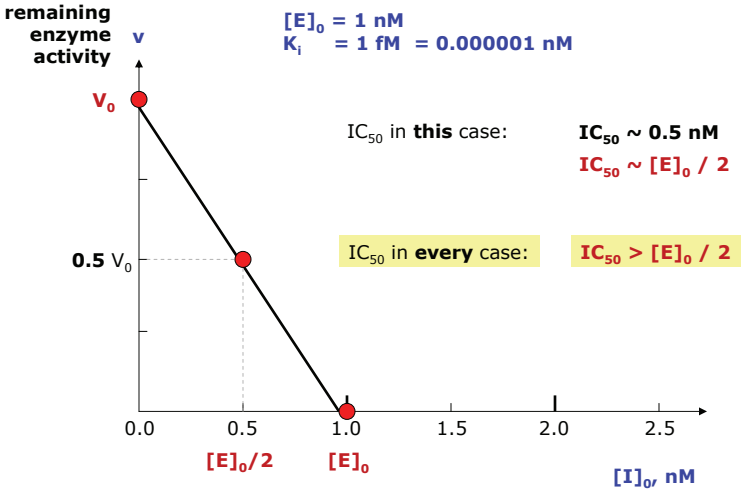
at **nano**molar $[E]_0 = [I]_0$ and **femto**molar K_i, there is **no free enzyme** left

A thought experiment: Titrate 1 nM enzyme, K_i = 0.000001 nM



Thought experiment, continued: What is the IC_{50} ?

CONCENTRATION OF INHIBITOR THAT PRODUCES **HALF-MAXIMUM** INHIBITORY EFFECT



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.

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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.

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What is "tight binding"

THERE IS NO SUCH THING AS A **"TIGHT BINDING INHIBITOR"**

Biochemical Pharmacology, Vol. 24, pp. 2177–2185.

TIGHT-BINDING INHIBITORS—I

KINETIC BEHAVIOR*

EXPERIMENTAL CONDITIONS

SUNGMAN CHA

Divison of Biological and Medical Sciences, Brown University, Providence, R.I. 02912, U.S.A.

(*Received* 30 *December* 1974; *accepted* 21 *February* 1975)

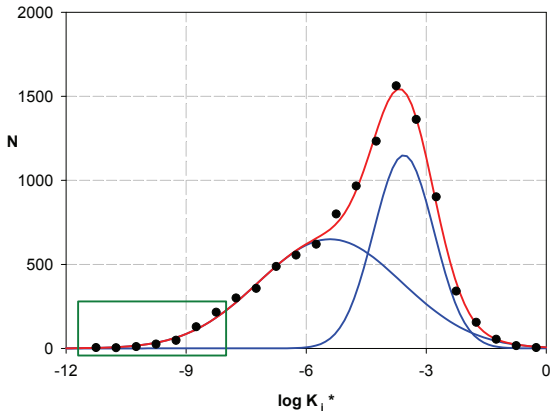
"Tight binding" kinetics applies when the enzyme concentration in any given assay is greater than the inhibition constant.

[E] / K_i ~ 1	tight binding is beginning to show up
[E] / K_i ~ 10	tight binding is definitely present
[E] / K_i ~ 100	highly tight binding
[E] / K_i ~ 1000	extremely tight binding

How prevalent is "tight binding" in a screening campaign?

EXAMPLE: A **PROTEASE** CAMPAIGN

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



about **4%** of compounds

Data courtesy of Celera Genomics 2003-2005

A word about the Cheng-Prusoff Equation

IT DOES **NOT** TAKE INTO ACCOUNT "TIGHT-BINDING"!

Cheng, Y.-Ch. and Prusoff, W. H. (1973)

"Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction"

Biochem. Pharmacol. **22**, 3099-3108.

competitive inhibition: $\mathbf{IC_{50} = K_i\ (1 + [S]/K_M)}$

Cha, S. (1975)

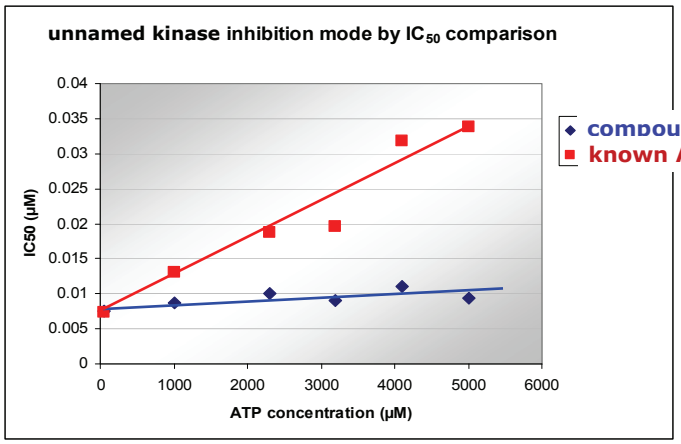
"Tight binding inhibitor. I. Kinetic behavior"

Biochem. Pharmacol. **24**, 2177-2185.

competitive inhibition: $\mathbf{IC_{50} = K_i\ (1 + [S]/K_M)} + [E]_0 / 2$

Many *J. Med. Chem.* papers still use the Cheng-Prusoff equation.
If the conditions are tight binding ([E] > K_i) this produces wrong K_i's.

Misuse of the "fold increase" plot: A case study



CONCLUSION: Compound "X" is an **ATP insensitive** inhibitor

not

A word about the "fold increase in IC_{50}" plot

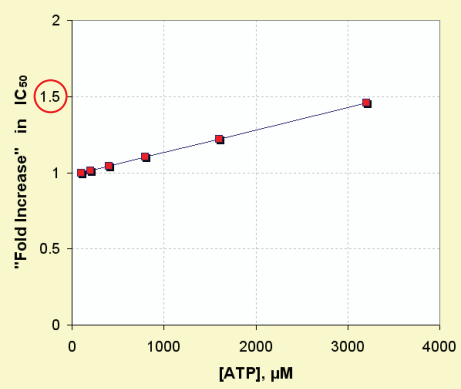
IT CAN BE **VERY** MISLEADING – **IF** "TIGHT BINDING" DOES OCCUR

SIMULATED KINASE EXAMPLE:

- **competitive**
- $K_M^{(ATP)}$ = 100 μM
- **K_i = 0.5 nM**
- **$[E]_0$ = 66 nM**

Very shallow slope, "unexpected".

$[E]_0$ >> Ki.



Another word about the "fold increase in IC_{50}" plot

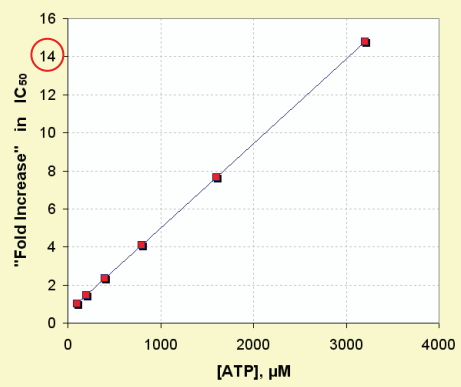
SOMETIMES IT DOES WORK, BUT ... you don't know **if** there is "tight binding" until you get the **$K_i^{(app)}$**

SIMULATED KINASE EXAMPLE:

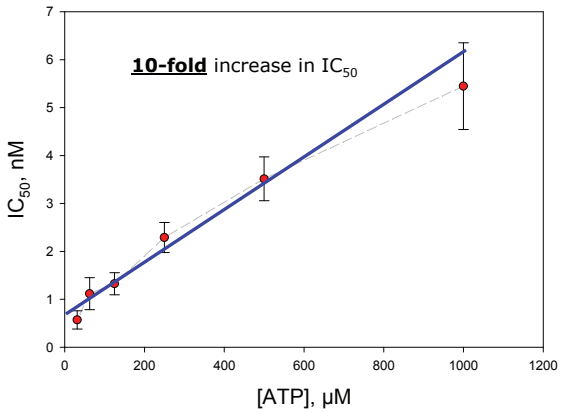
- **competitive**
- $K_M^{(ATP)}$ = 100 μM
- **K_i = 0.5 nM**
- **$[E]_0$ = 0.25 nM**

Steep slope, as "expected"

$[E]_0$ < Ki.



Compound "X" is an ATP-competitive inhibitor after all



Rules of thumb do not always work

What is the "IC_{50} Rule of Thumb":
IC_{50} for a ***competitive*** inhibitor should **increase about 10×** going from [ATP] = K_m to physiological [ATP].

What is "Tight Binding":
Experimental **conditions** where the **enzyme concentration** is comparable with the **inhibition constant**.

An important fact:
The "Rule of Thumb" does not apply under the conditions of "Tight Binding".

How do we know this:
Many journal articles on "Tight Binding" : Morrison (1969), Cha (1974), ..., Kuzmic (2000, 2003, 2011)

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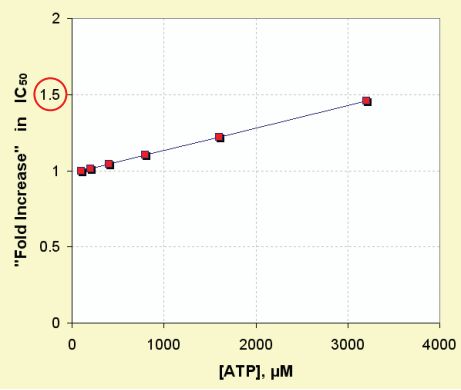
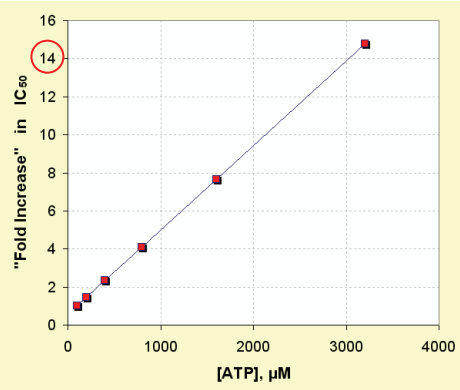
Final word about the "fold increase in IC_{50}" plot: Do not use it.

SOMETIMES IT WORKS AND SOMETIMES IT DOESN'T. **NO WAY OF TELLING** IN ADVANCE IF IT WILL.

$[E]_0$ = 0.25 nM



$[E]_0$ = 66 nM



SAME INHIBITOR – DIFFERENT ASSAY **CONDITIONS**

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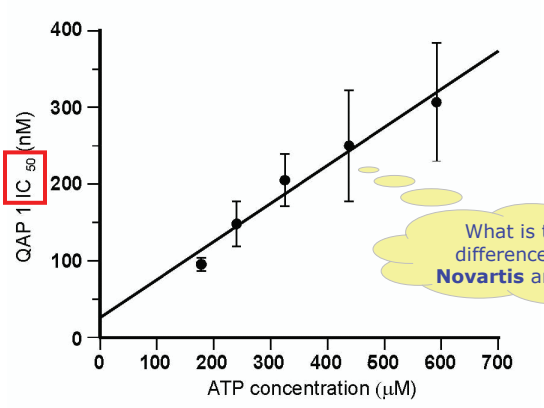
Other people still use the "fold increase in IC_{50}" plot

A RECENT PAPER FROM **NOVARTIS**



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

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Review: Measures of inhibitory potency

THE **INHIBITION CONSTANT** IS AN **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: $[E] \approx K_i$: $IC_{50} \neq K_i^*$

Summary: IC_{50}'s are bad for your drug discovery efforts

1. **You can hit the "IC_{50} Wall"**

   You could be looking at **picomolar K_i** inhibitors and yet they would look "**nanomolar**" to you, if you are screening at nanomolar [E].
   A thousand-fold difference in true vs. "apparent" potency.

2. **You could get very confused about the "mode" of inhibition**

   A **competitive** inhibitor can give you almost **no "fold increase"** in IC_{50} if the experiment is done under tight binding conditions.

3. **Muddled communication channels within your enterprise**

   The question "What is the biochemical IC_{50} for compound X?" **does not make sense** for a *competitive* inhibitor, **without specifying [ATP] level**.

➡ **Don't use IC_{50}** as measure of potency in **biochemical** assays.

IC_{50}'s are **perfectly good** for **cell-based** assays.

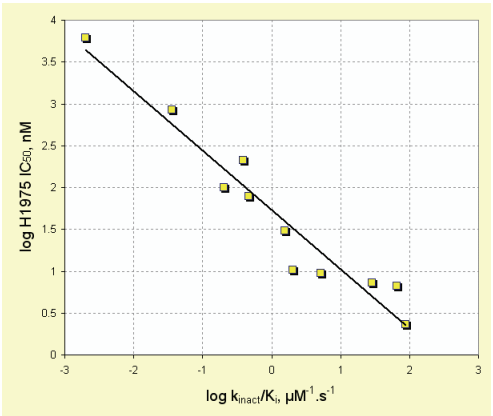
What else is there, other than the IC_{50}?

Use *intrinsic molecular measures* of potency (K_i, ...)

Example: Correlation between k_{inact}/K_i and cellular IC_{50}

"Molecular Underpinnings of Covalent EGFR Inhibitor Potency [...]" SUBMITTED

inhibition of EGFR-L858R/T790M autophosphorylation in H1975 tumor cells



$K_i^{(app)}$ takes into account "tight binding"

IT IS **CLOSE** TO BEING AN **INTRINSIC**, MOLECULAR MEASURE OF POTENCY

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**	$IC_{50} = K_i (1 + [S]/K_M) + [E]/2$

What do we need to do to move from IC_{50} to $K_i^{(app)}$?

The most "obvious" method to get $K_i^{(app)}$ will not work

THIS DOES **NOT** WORK

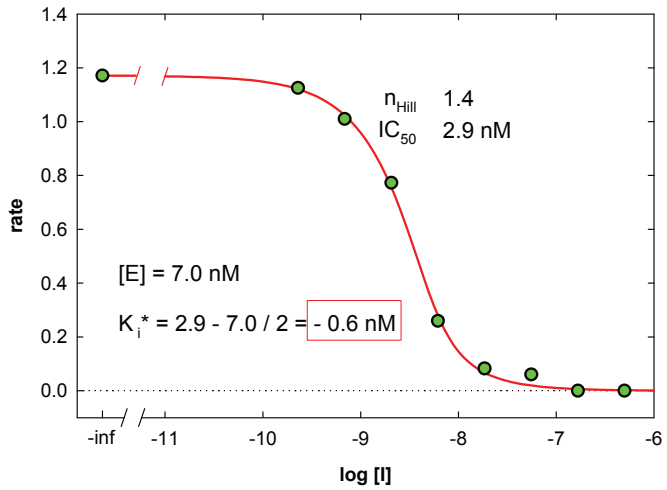
$$IC_{50} = \frac{[E]_0}{2} + K_i^{(app)} \quad \Rightarrow \quad K_i^{(app)} = IC_{50} - \frac{[E]_0}{2}$$

- Could we get the IC_{50} by our usual method, and then just subtract one half of $[E]_0$?
- Not in general:
  - This would work very well for **non-tight** situations, $[E]_0 << K_i^{(app)}$
  - In **non-tight** situation we have **$IC_{50} = K_i^{(app)}$**
  - However under **tight-binding** the $K_i^{(app)}$ could become **"negative"** if our enzyme concentration is lower than we think it is.

Problem: Negative K_i from IC_{50}

FIT TO FOUR-PARAMETER LOGISTIC:

$K_i^* = IC_{50} - [E] / 2$

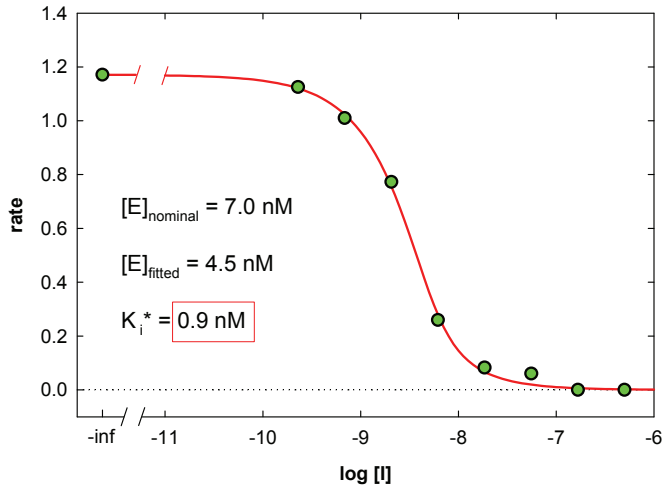


Data courtesy of Celera Genomics

Solution: Do not use four-parameter logistic equation

FIT TO MODIFIED MORRISON EQUATION:

P. Kuzmic *et al.* (2000) *Anal. Biochem.* 281, 62-67.
P. Kuzmic *et al.* (2000) *Anal. Biochem.* 286, 45-50.



Data courtesy of Celera Genomics

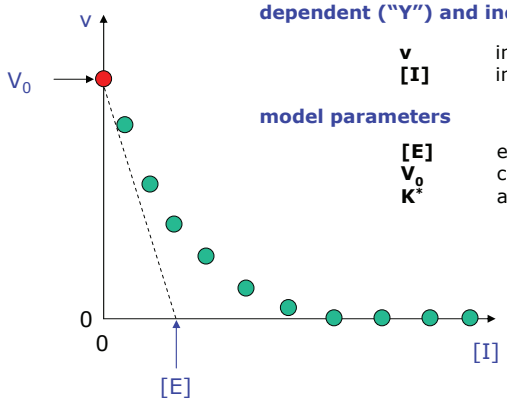
Surprise #1:

Getting the $K_i^{(app)}$ does <u>not</u> require any additional experiments

We just need to change the way the "IC_{50} data" are analyzed.

The "Morrison Equation" for tight binding inhibition

$$v = V_0 \frac{[E]-[I]-K^* + \sqrt{([E]-[I]-K^*)^2 + 4[E]K^*}}{2[E]}$$



dependent ("Y") and independent ("X") variables

v	initial reaction rate ("Y" axis)
[I]	inhibitor concentration ("X" axis)

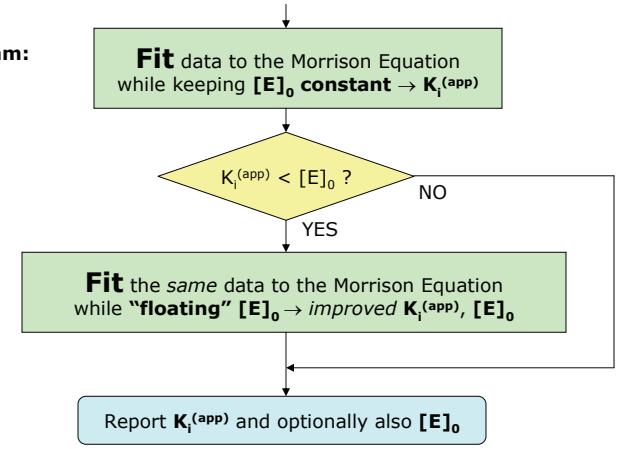
model parameters

[E]	enzyme concentration
V_0	control / uninhibited rate ([I] = 0)
K^*	apparent inhibition constant

Caveat: Sometimes we need to fit the same data twice

PROBLEM: WE NEVER QUITE KNOW WHAT THE ENZYME CONCENTRATION REALLY IS

Algorithm:



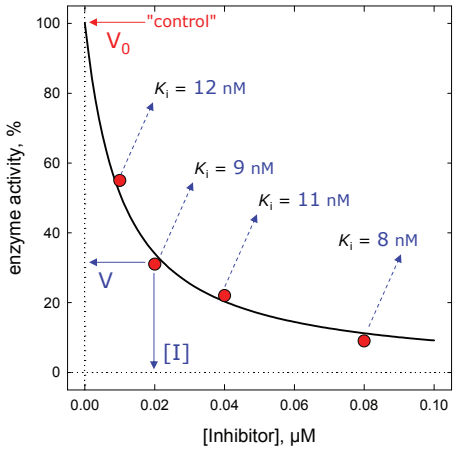
Kuzmic, P. *et al.* (2000) *Anal. Biochem.* **286**, 45-50.

Surprise #2:

$K_i^{(app)}$ can be guessed from a single concentration plus control

The "single-point" method for $K_i^{(app)}$

AN **APPROXIMATE** VALUE OF THE INHIBITION CONSTANT FROM A **SINGLE** DATA POINT



Relative rate

$V_r = V/V_0$

Single-point formula:

$$K_i = \frac{[I]-[E](V_r-1)}{1/V_r-1}$$

Kuzmic *et al.* (2000) *Anal. Biochem.* **281**, 62–67

Weighted average to make the *initial estimate* of $K_i^{(app)}$

DATA POINTS VERY NEAR "TOP" AND "BOTTOM" ARE LESS TRUSTWORTHY

$$K^* = \frac{[I]-[E](1-v'/V_z)}{V_z/v'-1}$$

Error Propagation Theory

$$\frac{\partial K}{\partial v} = \frac{K^* V_z/v'^2-[E]/V_z}{V_z/v'-1}$$

$$\frac{\partial K}{\partial V_z} = -\frac{K^*/v'+v'[E]/V_z^2}{V_z/v'-1}$$

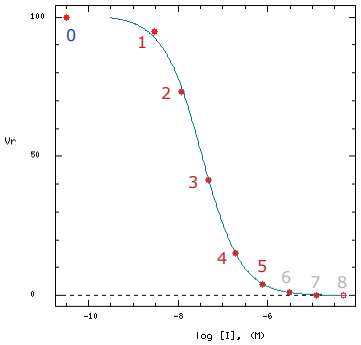
$$\frac{\partial K}{\partial [E]} = \frac{v'/V_z-1}{V_z/v'-1}$$

$$\Delta K^* = \sqrt{\Delta v'^2\left(\frac{\partial K}{\partial v}\right)^2+\Delta V_z^2\left(\frac{\partial K}{\partial V_z}\right)^2+\Delta [E]^2\left(\frac{\partial K}{\partial [E]}\right)^2}$$

Weight: $w = 1/\Delta K^*$

weighted average

Estimate *K**: Example



#	I, µM	rate', %	Ki, nM	weight
0	0	100.0		
1	0.003	94.8	54.6	0.04
2	0.012	73.2	32.6	0.29
3	0.049	41.6	34.4	0.39
4	0.195	15.1	34.5	0.22
5	0.781	4.3	34.9	0.07
6	3.125	1.2	--	0
7	12.5	0.0	--	0
8	50	0.1	--	0
wei. aver.			**34.7**	
best fit			**33.6**	

Weighted average.

- $[I]_{max}$ = 50 µM
- 4:1 dilution series
- 8 points + control

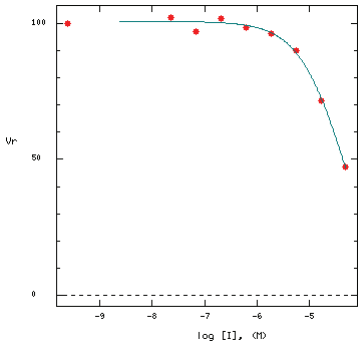
Surprise #3:

We don't need "fully developed" IC_{50} curves

... when using the Morrison Equation for $K_i^{(app)}$

It's OK to have at most 20%-30% inhibition if necessary

HIGHLY PRECISE $K_i^{(app)}$ EVEN WITH **LESS THAN 50% INHIBITION** AT MAXIMUM [INHIBITOR]



- $[I]_{max}$ = **50 µM**
- **4:1** dilution series
- **8** points + control

$K_i^{(app)}$ = (43 ± 2) µM

Can we do even better than $K_i^{(app)}$?

Getting the "true" K_i from a single dose-response curve

IF POSSIBLE, SCREEN AT **[SUBSTRATE] MUCH LOWER THAN THE MICHAELIS CONSTANT**

DEPENDENCE ON EXPERIMENTAL CONDITIONS	Depends on [S]	Depends on [E]	**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**	$IC_{50} = K_i (1 + [S]/K_M) + [E]/2$

At [S] << K_M ... $K_i^{(app)} \approx K_i$

Getting the "true" K_i from a single dose-response curve

FOR **"NONCOMPETITIVE"** INHIBITORS $K_i^{(app)} = K_i^{(true)}$ ALWAYS

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

Summary and Conclusions

- Biochemical **IC_{50}'s** are misleading in multiple ways.
- They are a relic of previous eras (1950-1980) in pre-clinical research.
- Even *Big Pharma* is now gradually moving toward **$K_i^{(app)}$** and **K_i**.
- Small to medium-size companies should not "follow the Big Guys".

  *They **should lead.***