Irreversible Inhibition Kinetics:
Biochemical Rate Constants *vs.* Cell-based IC_{50}

Petr Kuzmič, Ph.D.
BioKin, Ltd.

1. EGFR inhibition by covalent drugs (*PNAS*, January 2014)
2. New results using previously published data
3. PK/PD simulations

BioKin L t d.

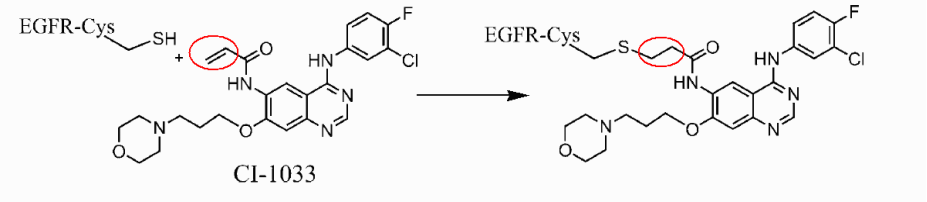
EGFR inhibition by covalent drugs

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

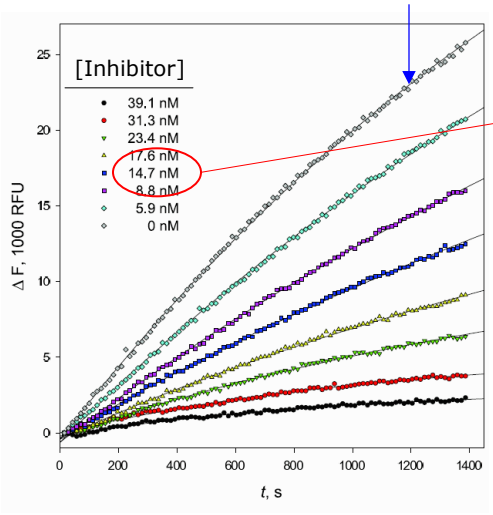
EXAMPLE:



Example data: Neratinib vs. EGFR T790M / L858R mutant

OBSERVE FLUORESCENCE INCREASE OVER TIME

nonlinear "control" progress curve



[Enzyme] = 13 nM

"tight binding" inhibition

Conventional kinetic analysis of covalent inhibition

TWO-STEP ALGEBRAIC METHOD

1. Fit [Product] vs. time to obtain k_{obs}
2. Fit k_{obs} vs. [Inhibitor] to obtain k_{inact} and K_i

THIS METHOD RELIES ON TWO IMPORTANT **ASSUMPTIONS**

1. Control progress curve ($[I]_0 = 0$) is strictly linear

   Implies near zero substrate consumption or else $[S]_0 >> K_M$

2. Inhibitor is *not* "tight binding"

   Implies $[E]_0 << K_i$

Generalized numerical kinetic analysis of covalent inhibition

SINGLE-STEP NUMERICAL METHOD

- Global fit of [Product] vs. time to obtain microscopic rate constants
- Numerical-mathematical model is a system of differential equations
- The model is derived automatically using the software *DynaFit*

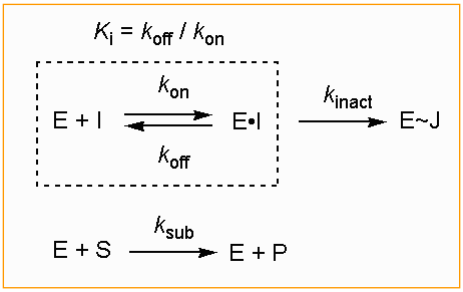
Kuzmic, P. (2009)

"*DynaFit* – A software package for enzymology" [a review]

Methods in Enzymology **467**, 247-280.

EGFR inhibition by covalent drugs: Mechanistic model

THREE STEPS IN THE INHIBITION BRANCH OF OVERALL MECHANISM



THREE STEPS:

1.	k_{on}	association	assumed to be extremely rapid
2.	k_{off}	dissociation	
3.	k_{inact}	inactivation	

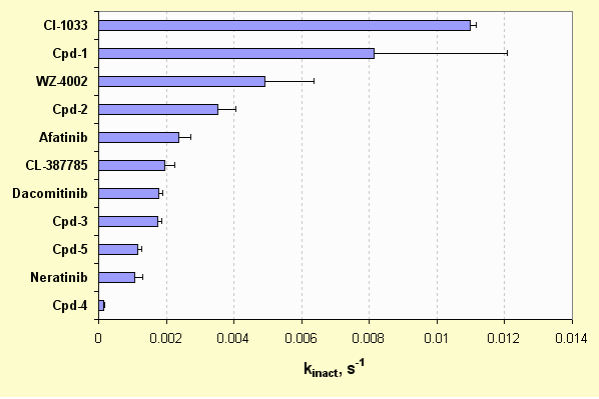
EGFR inhibition by covalent drugs: Results

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} EI \xrightarrow{k_{inact}} E\sim I$$

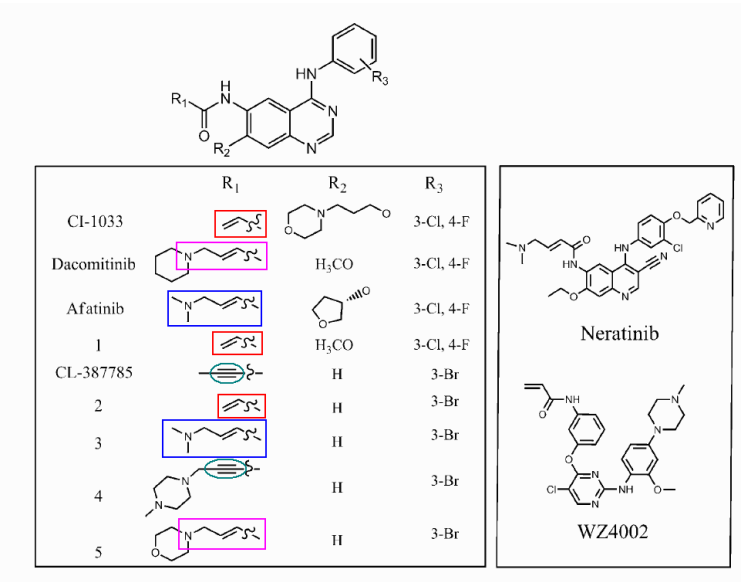
Compound	1000 k_{inact}, s^{-1}	±SD	K_i, nM	±SD
Afatinib	2	0.3	2.8	0.6
CI-1033	11	0.2	1.9	0.4
CL-387785	2	0.3	180	40
Cpd-1	8	4	2	1
Cpd-2	4	0.6	40	5
Cpd-3	2	0.1	70	20
Cpd-4	0.2	0.02	1800	300
Cpd-5	1.2	0.1	500	40
Dacomitinib	1.8	0.1	10.7	0.9
Neratinib	1.1	0.2	2.4	0.5
WZ-4002	5	2	230	50

Chemical reactivity distribution

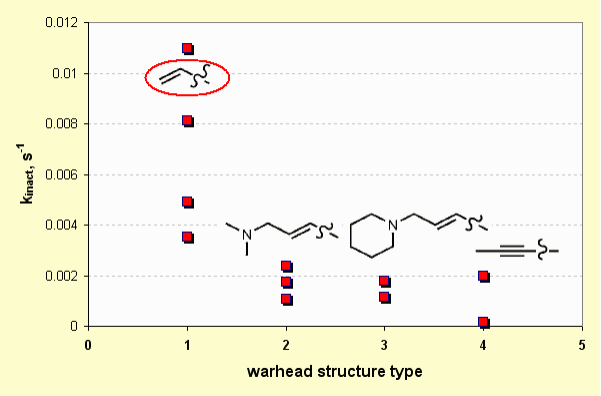
REACTIVITY VARIES BY TWO TO THREE ORDERS OF MAGNITUDE



Small number of warhead structures in the test panel



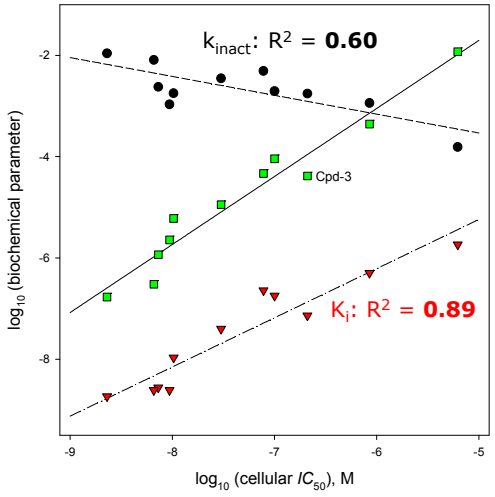
Warhead structure type vs. inactivation reactivity



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

Biochemical vs. cellular potency: Summary

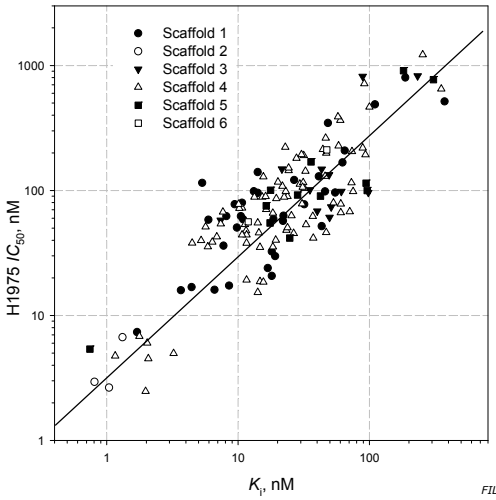
INITIAL (NON-COVALENT) BINDING SEEMS MORE IMPORTANT THAN CHEMICAL REACTIVITY



FILE: cell-IC50-001.JNB (9/29/2013)

Cellular potency: Importance of non-covalent binding

154 EGFR (W.T.) INHIBITORS ACROSS SIX STRUCTURAL SCAFFOLDS



FILE: ki-cell-ic50-large.JNB (10/3/2013)

EGFR inhibition by covalent drugs: Summary

1. Both binding and reactivity are important for cellular potency
2. Initial binding seems more important by R^2 test
3. Chemical structure of warhead has only minor effect on k_{inact}
   - Wide variation of k_{inact} for the same structure
   - Similar k_{inact} for different warhead structures

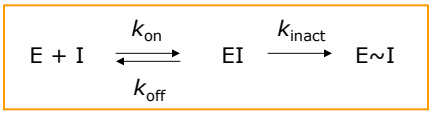
Warhead alone is not a silver bullet.
Waht matter is the *balance* between binding and reactivity.

Irreversible Inhibition Kinetics:
Biochemical Rate Constants *vs.* Cell-based IC_{50}

Petr Kuzmič, Ph.D.
BioKin, Ltd.

1. EGFR inhibition by covalent drugs (*PNAS*, January 2014)
2. New results using previously published data
3. PK/PD simulations

A deeper look at enzyme-inhibitor interactions



THREE STEPS:

1. k_{on} association
2. k_{off} dissociation
3. k_{inact} inactivation

Can we pick these two apart?

Confidence interval method

DETAILS NOT SHOWN – MANUSCRIPT IN PREPARATION

OUTLINE:

- We cannot get "best-fit" values of k_{on} and k_{off} separately
- However, we can get the lower limits for both k_{on} and k_{off}
- Monte-Carlo simulation: Lower limits correlate with "true" values

---

- Conclusion / Working Hypothesis:

  Lower limits on k_{on} and k_{off} are a good measure of **"true" values**

Results: Lower limits for k_{on} and k_{off}

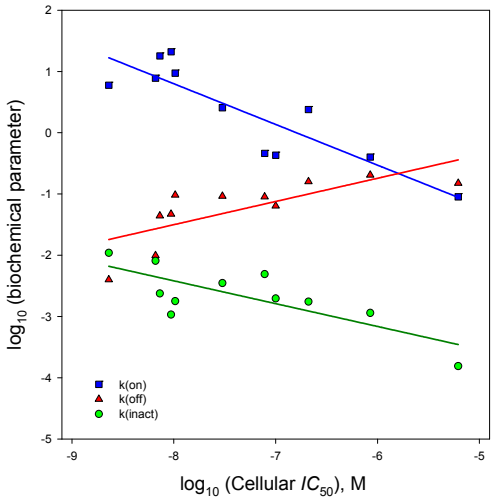
$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EI \xrightarrow{k_{inact}} E{\sim}I$$

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

Compound	k_{on}, μM^{-1} s^{-1}	±SD	k_{off}, s^{-1}	±SD	1000 k_{inact}, s^{-1}	±SD
Afatinib	18	5	0.044	0.003	2.4	0.3
CI-1033	6	1	0.004	0.002	11	0.2
CL-387785	0.4	0.3	0.06	0.04	2	0.3
Cpd-1	8	4	0.01	0.002	8	4
Cpd-2	2.6	0.3	0.092	0.003	4	0.6
Cpd-3	2.4	0.7	0.16	0.02	2	0.1
Cpd-4	0.09	0.02	0.15	0.01	0.2	0.02
Cpd-5	0.4	0.3	0.2	0.2	1.2	0.1
Dacomitinib	9.4	0.6	0.096	0.004	1.8	0.1
Neratinib	21	4	0.047	0.002	1.1	0.2
WZ-4002	0.5	0.2	0.09	0.02	5	2

Biochemical vs. cellular potency

ASSOCIATION RATE CONSTANT SEEMS MORE IMPORTANT THAN **DISSOCIATION**



k_{off}: R^2 = **0.56**

k_{on}: R^2 = **0.77**

k_{inact}: R^2 = **0.60**

FILE: cell-IC50-001.JNB (9/29/2013)

Biochemical vs. cellular potency: Revised summary

DETAILED ANALYSIS: SEPARATELY EVALUATING ALL THREE MICROSCOPIC STEPS

- Both binding and reactivity are important for cellular potency
- Binding should be dissected into (a) association and (b) dissociation
- Association seems more important than dissociation
- Relative order of importance in determining **cellular** IC_{50}:

  1. association (R^2 ~ 0.**8**)

  2. dissociation (R^2 ~ 0.**6**) ~ reactivity (R^2 ~ 0.**6**)

Irreversible Inhibition Kinetics:
Biochemical Rate Constants *vs.* Cell-based IC_{50}

Petr Kuzmič, Ph.D.
BioKin, Ltd.

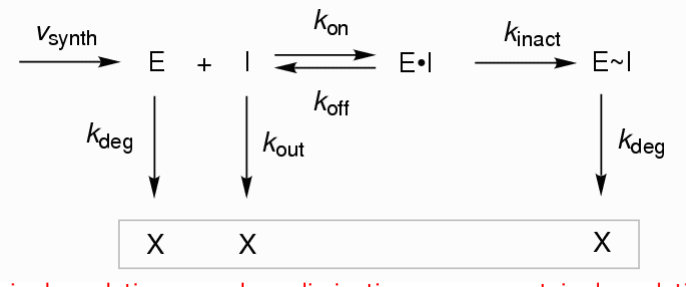
1. EGFR inhibition by covalent drugs (*PNAS*, January 2014)
2. New results using previously published data
3. PK/PD simulations

BioKin Ltd.

Possible cellular mechanism

REALISTIC PK/PD MODEL MUST ACCOUNT FOR METABOLISM OF PROTEIN AND DRUG MOLECULES

protein re-synthesis



protein degradation drug elimination protein degradation

Possible cellular mechanism in DynaFit software

DYNAFIT USES "SYMBOLIC" REPRESENTATION OF ARBITRARY MOLECULAR MECHANISM

Example DynaFit input:

```
[task]

   task = simulate
   data = progress

[mechanism]

   E + I <==> E.I    :    kon      koff
     E.I ---> E~I    :    kinact

       I ---> X      :    kout
         ---> E      :    ksyn
       E ---> X      :    kdeg
     E~I ---> X      :    kdeg

...
```

Possible cellular mechanism in DynaFit software (cont.)

RATE CONSTANTS AND CONCENTRATIONS MUST BE GIVEN CONSISTENT UNITS

Example DynaFit input (*continued*):

```
...

[constants]  ;  units µM, sec

   kon    = 1
   koff   = 0.01
   kinact = 0.001

   kout   = 0.0000641803        ; 3 h drug half-life
   ksyn   = 0.000000001605      ; 0.0001 uM per 12 h * ln(2)
   kdeg   = 0.00001605          ; 12 h protein half-life

...
```

properties of a hypothetical inhibitor

Possible cellular mechanism in DynaFit software (cont.)

RATE CONSTANTS AND CONCENTRATIONS MUST BE GIVEN CONSISTENT UNITS

Example DynaFit input (*continued*):

```
...

[concentrations]  ;  units µM

   E = 0.0001

[responses]

   E = 1000000    ; 100% free protein at time zero

...
```

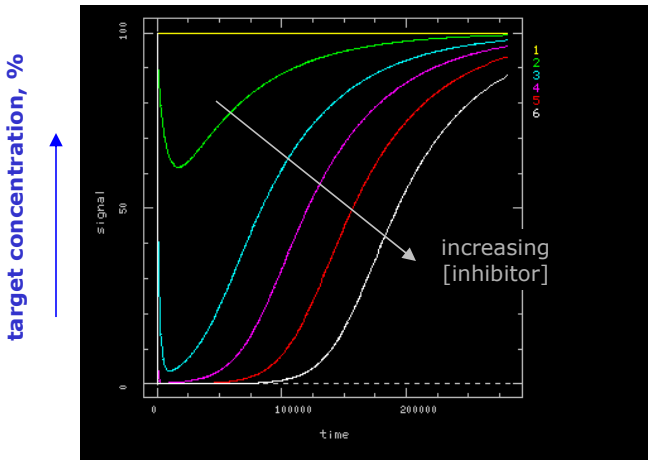
Possible cellular mechanism in DynaFit software (cont.)

RATE CONSTANTS AND CONCENTRATIONS MUST BE GIVEN CONSISTENT UNITS

Example DynaFit input (*continued*):

```
...

[data]

   mesh        linear from 1 to 259200 step 600
   directory   ./users/COM/Pfizer/140311/data/sim-003
   extension   txt

   file i00   | concentration I = 0
   file i01   | concentration I = 0.0001
   file i02   | concentration I = 0.001
   file i03   | concentration I = 0.01
   file i04   | concentration I = 0.1
   file i05   | concentration I = 1

...
```


DynaFit simulation output: Afatinib – strong inhibitor

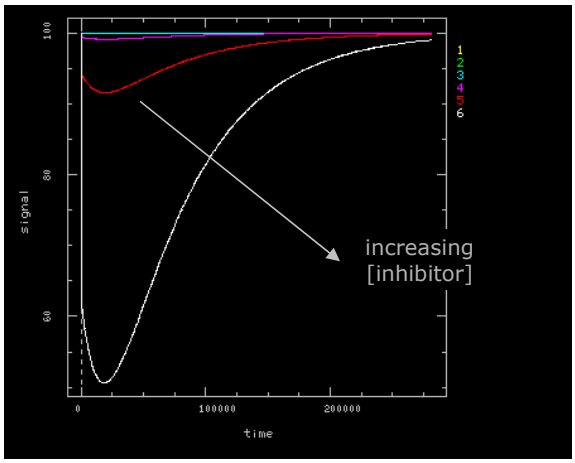


Afatinib:

```
kon    = 18
koff   = 0.044
kinact = 0.0024
```

DynaFit simulation output: Compound 4 – weak inhibitor

target concentration, %



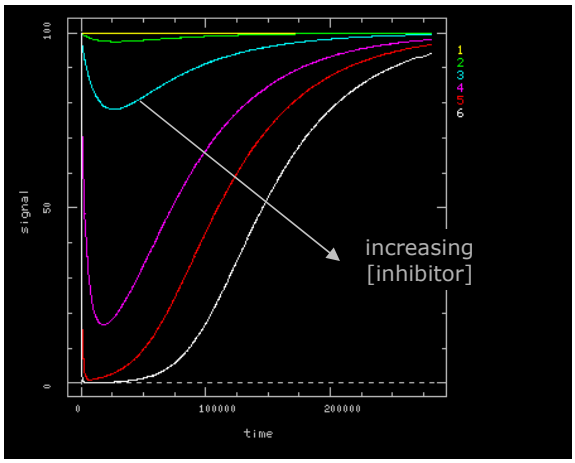
time, seconds (total = 72 hours)

Compd. 4:

```
kon    = 0.09
koff   = 0.15
kinact = 0.00015
```


DynaFit simulation output: Compound 3 – intermediate inhibitor

target concentration, %

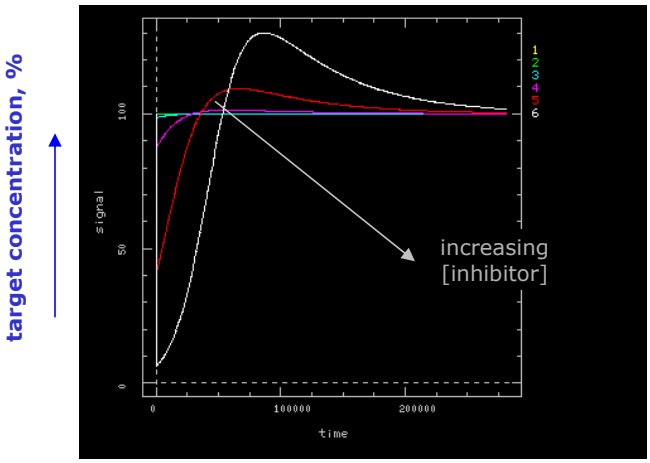


time, seconds (total = 72 hours)

Compd. 3:

```
kon    = 2.4
koff   = 0.16
kinact = 0.0018
```

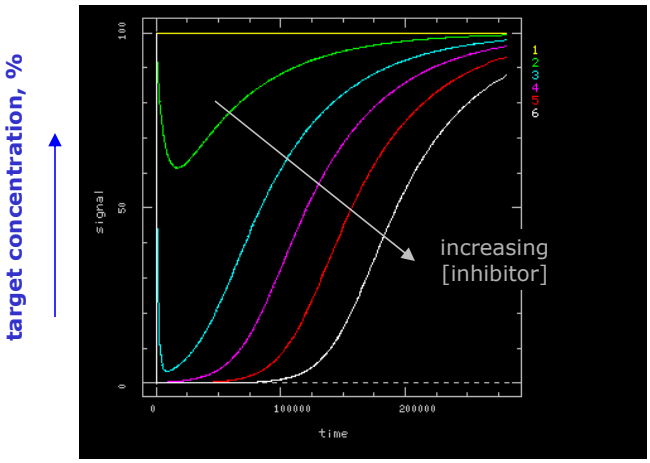
DynaFit simulation output: "Like" compound 3 – zero inactivation



kon = 2.4
koff = 0.16
kinact = ~ 0

time, seconds (total = 72 hours)

DynaFit simulation output: "Like" compound 3 – high inactivation

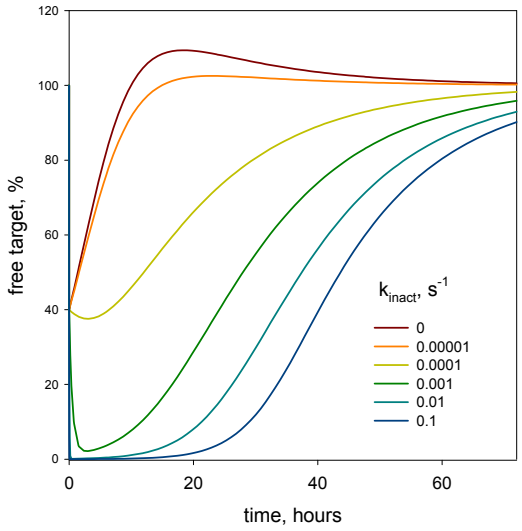


kon = 2.4
koff = 0.16
kinact = 0.1

time, seconds (total = 72 hours)

"Like" compound 3: k_{inact} vs. free [target]

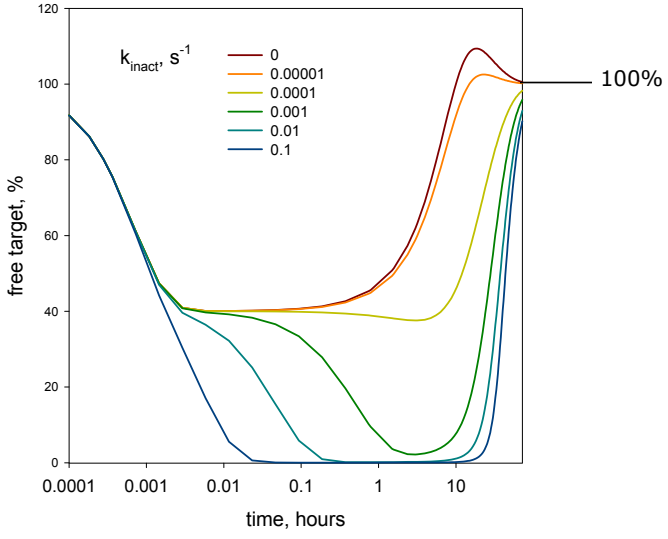
SIMULATION STUDY: EFFECT OF **INACTIVATION** RATE CONSTANT



BioKin Ltd.

"Like" compound 3: k_{inact} vs. free [target]

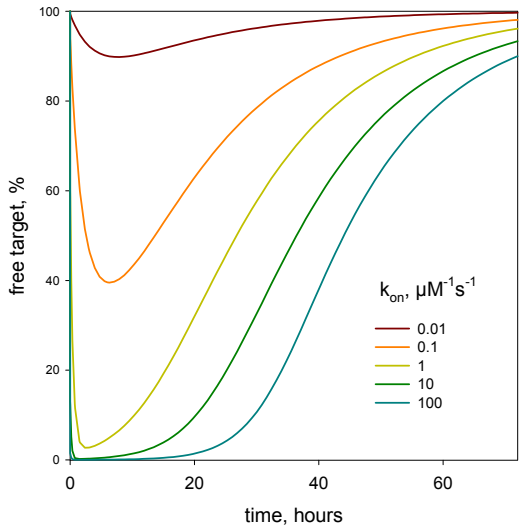
SIMULATION STUDY: EFFECT OF **INACTIVATION** RATE CONSTANT



BioKin Ltd.

"Like" compound 3: k_{on} vs. free [target]

SIMULATION STUDY: EFFECT OF **ASSOCIATION** RATE CONSTANT

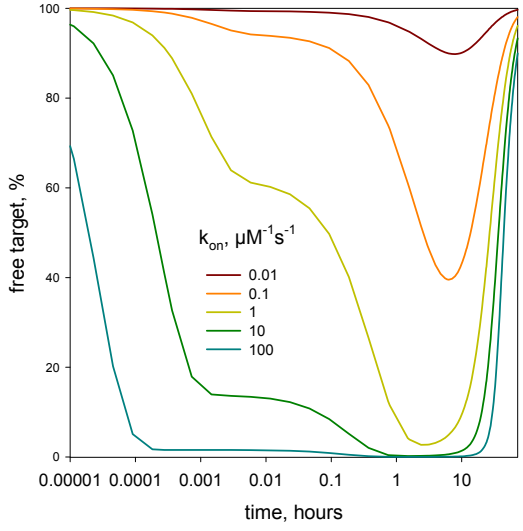


BioKin Ltd.

33

"Like" compound 3: k_{on} vs. free [target]

SIMULATION STUDY: EFFECT OF **ASSOCIATION** RATE CONSTANT



BioKin Ltd.

34

PK / PK simulations: Summary and conclusions

- Both binding and reactivity are important for cellular potency
- Binding is a **necessary but not sufficient** precondition
- Reactivity is a **necessary but not sufficient** precondition
- The same target suppression can be achieved in two different ways:

1. Have a highly "sticky" molecule, no matter how reactive
   (could be very un-reactive but if it really "sticks", it will do the job)
2. Have a highly reactive molecule, but it must be at least a little "sticky"
   (if the molecule does not "stick" at all, it does no matter how "hot" the warhead is)

All these things can be better understood and fine-tuned with a tool like DynaFit.

Acknowledgments

- Brion Murray – Pfizer
  Leader on the PNAS paper, and in other ways
- Art Wittwer – Confluence Technologies (formerly Pfizer)
  PK/PD initial scripts (and many other ideas)
- Phillip Schwartz – Takeda (formerly Pfizer)
  Data collection for EGFR inhibitors

Questions ?

http://www.**biokin**.com