Using the Caliper Technology for Automated Mechanism of Action Assessment of Kinase inhibitors

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Abstract:

We have used the Caliper method (off-chip incubation mode) to study the inhibition mechanism of kinase inhibitors (ATP competitive, non-competitive, and uncompetitive). The technology is homogeneous and provides high quality data. In addition, there is no upper limit on the ATP concentration that can be used in the assay. 10 concentrations of kinase inhibitor have been tested to determine IC_{50}s at 8 different ATP concentrations ranging from approx. 200 Km to 10 Km. This setup allows to measure two compounds in duplicate on one 384 well plate. The liquid handling process is semi-automated. The test data are analyzed with global fitting whereby all data sets are fitted with shared kinetic parameters (K_i, K_{ii}).

Rationale

- A limitation of primary HTS results is in IC_{50} at one [S] which is not immediately convertible into Ki.
- Most of ATP competitive kinase inhibitors have problems with selectivity and patent ability.
- The Kinase inhibitors identified under low [ATP] in HTS have low potency in cells (high [ATP]).

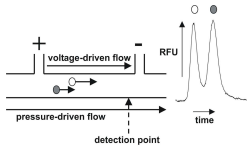
Therefore non-competitive or uncompetitive compounds with high IC_{50} are potentially more valuable than low IC_{50} competitive inhibitors.

Goals:

⇒determine K_i and mode of action for interesting hit candidates
⇒automate the MOA process from liquid handling to data analysis

Principle:

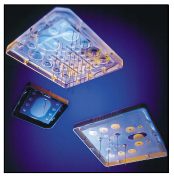
1.Caliper mobility-shift (off-chip) assay



substrate: fluorescein-RPRAATF, q= ±0
product: fluorescein-RPRAA(pT)F, q= -2

Caliper 3000 and chips (12-sipper and 4 sipper)

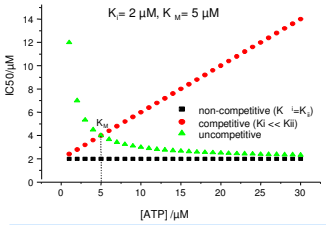
LabChip 3000
Drug Discovery System



Caliper technology: chip-based microfluidic method. Differently charged substrate (peptide) and product have different migration rate in the electronic field on the chip. Fluorescence signal will be measured. The readout is ratiometric; substrate and product are quantitatively determined .

2.Mechanism of Action

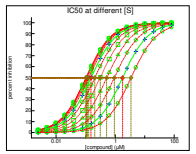
Relationship between K_i and IC_{50} (3)

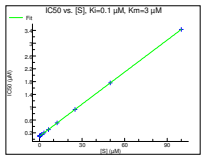


K_i - *dissociation constant* of enzyme-inhibitor complex
IC_{50} - concentration of inhibitor lowering enzymatic activity to 50 %
Thus, K_i is *independent* of [substrate] whereas IC_{50} is not.

3.Data analysis with global fit

all data sets are fitted with shared kinetic parameters K_i and K_{ii}





$$\%Inh = Y_{min} + \frac{Y_{max} - Y_{min}}{1 + \left(\frac{IC50}{[I]}\right)^{nH}}$$

$$IC_{50} = \frac{K_M + [S]}{\frac{K_M}{K_i} + \frac{[S]}{K_{ii}}}$$

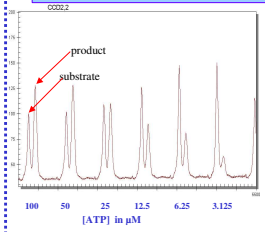
Global fit

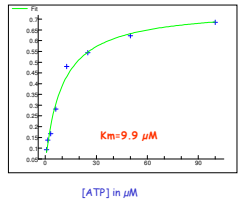
$$\%\,Inh = Y_{min} + \frac{Y_{max} - Y_{min}}{1 + \left(\frac{\frac{K_M + [S]}{\frac{K_M}{K_i} + \frac{[S]}{K_{ii}}}}{[I]}\right)^{nH}}$$

Assay development and validation

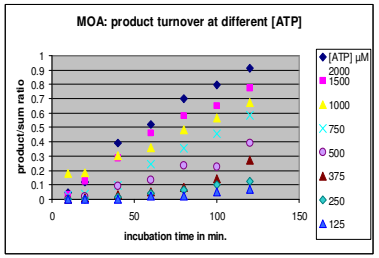
The kinase and peptide concentrations for the assay are optimized at a ATP concentration of 10 µM.
Under this assay condition the Km value of ATP was determined.

Km determination for ATP





Determination of incubation time for reaction sets at [ATP]s



The rate of product turnover is [substrate]-dependent.
This test determined the incubation time for the reaction sets at different [ATP]s.
The optimal product turnover should be 50% (product/sum ratio at 0.5).
The activities are showed as product/sum (product+substrate) ratio.

Compound Screening

Liquid handling and assay protocol:
Before the assay is started, the following plates are prepared on a Biomek workstation:

1. Compound plate: each compound on a 96-well plate diluted at 10 concentrations (in µM) from column 1 to 10.

	1	2	3	4	5	6	7	8	9	10	11	12
A	5	1.667	0.556	0.185	0.06	0.02	0.0069	0.002	0.0008	0.0003	buffer	buffer
B	5	1.667	0.556	0.185	0.06	0.02	0.0069	0.002	0.0008	0.0003	buffer	buffer
C	5	1.667	0.556	0.185	0.06	0.02	0.0069	0.002	0.0008	0.0003	buffer	buffer
D	5	1.667	0.556	0.185	0.06	0.02	0.0069	0.002	0.0008	0.0003	buffer	buffer
E	5	1.667	0.556	0.185	0.06	0.02	0.0069	0.002	0.0008	0.0003	buffer	buffer
F	5	1.667	0.556	0.185	0.06	0.02	0.0069	0.002	0.0008	0.0003	buffer	buffer
G	5	1.667	0.556	0.185	0.06	0.02	0.0069	0.002	0.0008	0.0003	buffer	buffer
H	5	1.667	0.556	0.185	0.06	0.02	0.0069	0.002	0.0008	0.0003	buffer	buffer

[compound]s in µM

2. ATP plate (in µM): on a 96-well plate 8 [ATP]s are from rows A to H

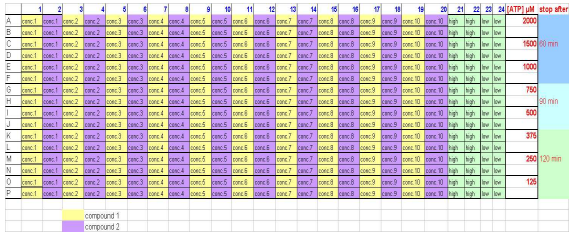
	1	2	3	4	5	6	7	8	9	10	11	12
A	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000	buffer
B	1500	1500	1500	1500	1500	1500	1500	1500	1500	1500	1500	buffer
C	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	buffer
D	750	750	750	750	750	750	750	750	750	750	750	buffer
E	500	500	500	500	500	500	500	500	500	500	500	buffer
F	375	375	375	375	375	375	375	375	375	375	375	buffer
G	250	250	250	250	250	250	250	250	250	250	250	buffer
H	125	125	125	125	125	125	125	125	125	125	125	buffer

3. One 384-well plate with enzyme
4. One 384-well plate with FL-peptide

Assay protocols: using Biomek work station the following reagents are added on a black 384-well test plates
3 µl compounds (Biomek FX 96-tip head)
3 µl enzyme, 15 min preincubation (Biomek FX 384-tip head)
3 µl FL-peptide (Biomek FX 384-tip head)
3 µl ATP (Biomek FX 96-tip head)
Incubation and stop the assays with 10 µl stop buffer
Measurement on Caliper

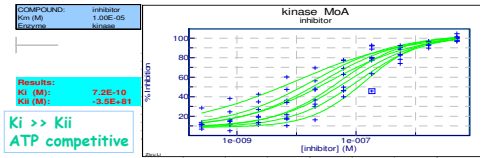
Test plate layout

On each 384-well test plate two compounds are tested in duplicate.



Automatic data analysis with global fit

The shared ki and Kii are fitted global with the 8 data sets (8 ATP concentrations)



Ki >> Kii
ATP competitive

Compound (µM)	ATP (M) 0.002	0.0015	0.001	0.00075	0.0005	0.000375	0.00025	0.000125
0.000005	100.8	96.5	104.6	99.5	97.9	97.1	100.7	101.2
1.6867E-06	96.8	89.6	96.3	92.6	95.7	93.0	95.4	96.2
5.5556E-07	78.2	73.9	82.5	83.2	88.2	90.2	90.7	92.4
1.8519E-07	45.6	46.8	63.4	80.1	77.2	79.7	92.5	92.0
6.1728E-08	46.2	40.0	46.1	49.5	68.8	62.9	77.5	77.1
2.0576E-08	16.1	29.6	32.4	36.6	47.0	47.9	56.3	69.4
6.8587E-09	10.2	17.0	21.6	18.0	36.3	33.8	47.3	60.2
2.2862E-09	11.1	13.7	19.9	13.4	26.9	18.9	34.7	42.3
7.6208E-10	5.1	11.2	12.0	15.3	16.8	14.6	24.3	38.1
2.5403E-10	9.1	11.1	11.4	6.8	13.6	12.1	11.5	28.4
IC50 (M)	1.4E-07	1.1E-07	7.2E-08	5.4E-08	3.6E-08	2.8E-08	1.9E-08	9.7E-09
Ymin	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4
Ymax	103.5	103.5	103.5	103.5	103.5	103.5	103.5	103.5
Km (M)	0.00001	0.00001	0.00001	0.00001	0.00001	0.00001	0.00001	0.00001
S (M)	0.002	0.0015	0.001	0.00075	0.0005	0.000375	0.00025	0.000125
Ki (M)	7.15E-10	7.15E-10	7.15E-10	7.15E-10	7.15E-10	7.15E-10	7.15E-10	7.15E-10
Kii (M)	-3.5E+81	-3.5E+81	-3.5E+81	-3.5E+81	-3.5E+81	-3.5E+81	-3.5E+81	-3.5E+81
nH	0.9	0.8	0.7	0.8	0.8	0.8	0.8	0.8
Fit statistics	#Ok	#Ok	#Ok	#Ok	#Ok	#Ok	#Ok	#Ok
Global Fit	#Ok	5.72	9.92	1.77	4.99	4.37	1.76	4.61	3.26
		5.24						

Summary

- The caliper assay technology is ideally suitable for MOA study of kinase inhibitors because of its advantages of the rapid assay development, high sensitivity at low substrate turnover, high quality data and no upper limit on [ATP].
- At different substrate concentrations, the reaction rate / turnover is different, therefore different incubation times and/or enzyme concentrations are required for different substrate concentrations
- Liquid handling for compounds preparation as well as assay reaction is semi-automated. Two compounds can be tested in duplicate on a 384-well plate.50 compounds can be tested daily.
- With the global fitting analysis sheet the ki /kii of the inhibitor can be automatically calculated and the data quality is controlled through the IC50-curves.

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