A biochemical approach to discriminate between ATP-competitive and non-ATP-competitive protein kinase inhibitors

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Introduction

Protein kinases are central to cellular signal transduction and regulation of cellular processes, and are one of the most attractive target classes in modern drug discovery. Multiple kinase inhibitors have already been approved for treatment of various diseases including such severe conditions as cancer.

The first and up to today largest group of drugs that effectively inhibited their respective kinase targets belong to the class of ATP-competitive compounds. They bind into or near the ATP binding site of the enzymes and inhibit kinase activity by blocking access of ATP to the active site. Although there are numerous examples of highly specific ATP-competitive compounds this mode of action is limited by several factors: The ATP binding pocket structures of kinases show a high degree of similarity, which makes finding highly selective compounds challenging. Furthermore, competing with ATP for binding to the same target site, compounds have to be of very high affinity due to the high intracellular ATP concentrations.

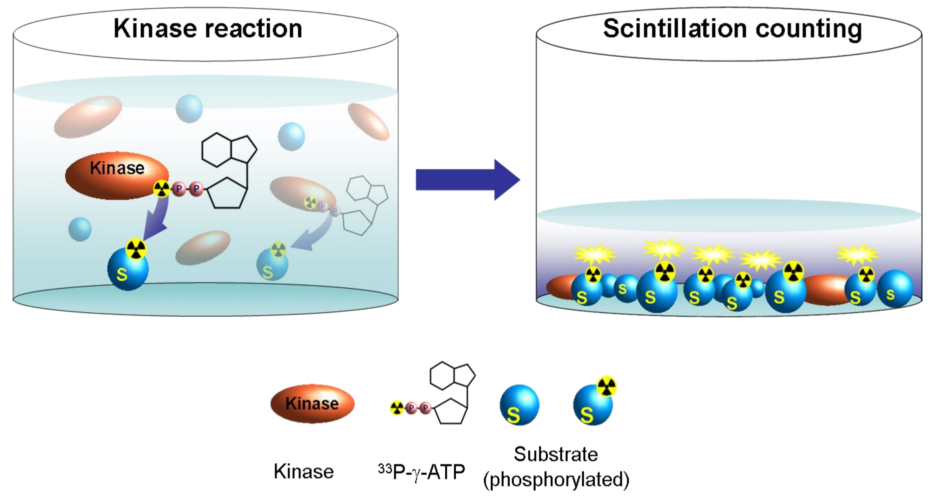
Therefore, the interest to develop non-ATP-competitive inhibitors has risen considerably over the last years. Such inhibitors bind to kinases at sites apart from the ATP binding site, inhibiting their activity e.g. by stabilizing an inactive conformation (like DFG-out state binders), displacing essential cofactors (like cyclins for CDKs) or by blocking activating modifications (like phosphorylation by upstream kinases). The IC_{50} values of ATP-competitive inhibitors apparently increase if the ATP concentration in the assay is increased. For a purely ATP-competitive inhibitor this change in the IC_{50} value may be calculated by the formula suggested by Cheng and Prusoff[1] :

$$IC_{50} = K_i\left(1 + \frac{[ATP]}{K_{M[ATP]}}\right)$$

By determining IC_{50} values for an inhibitor of a specific kinase at ATP concentrations ranging from $0.1xK_{M[ATP]}$ to $10xK_{M[ATP]}$ we examined whether the IC_{50} value changed according to the Cheng-Prusoff equation >10fold, indicating an ATP-competitive mode of action, or if the IC_{50} values remain unchanged or change only slightly in presence of elevated ATP indicating a non-ATP competitive or mixed type mode of action respectively.

By comparing the results for the non-ATP-competitive inhibitor selumetinib[2], the type-2 inhibitor sorafenib[3] and staurosporine[4,5] using different kinases, we could verify that our assay setup is well suited to discriminate kinase inhibitors with regard to their ATP competition characteristics.

Assay Principle



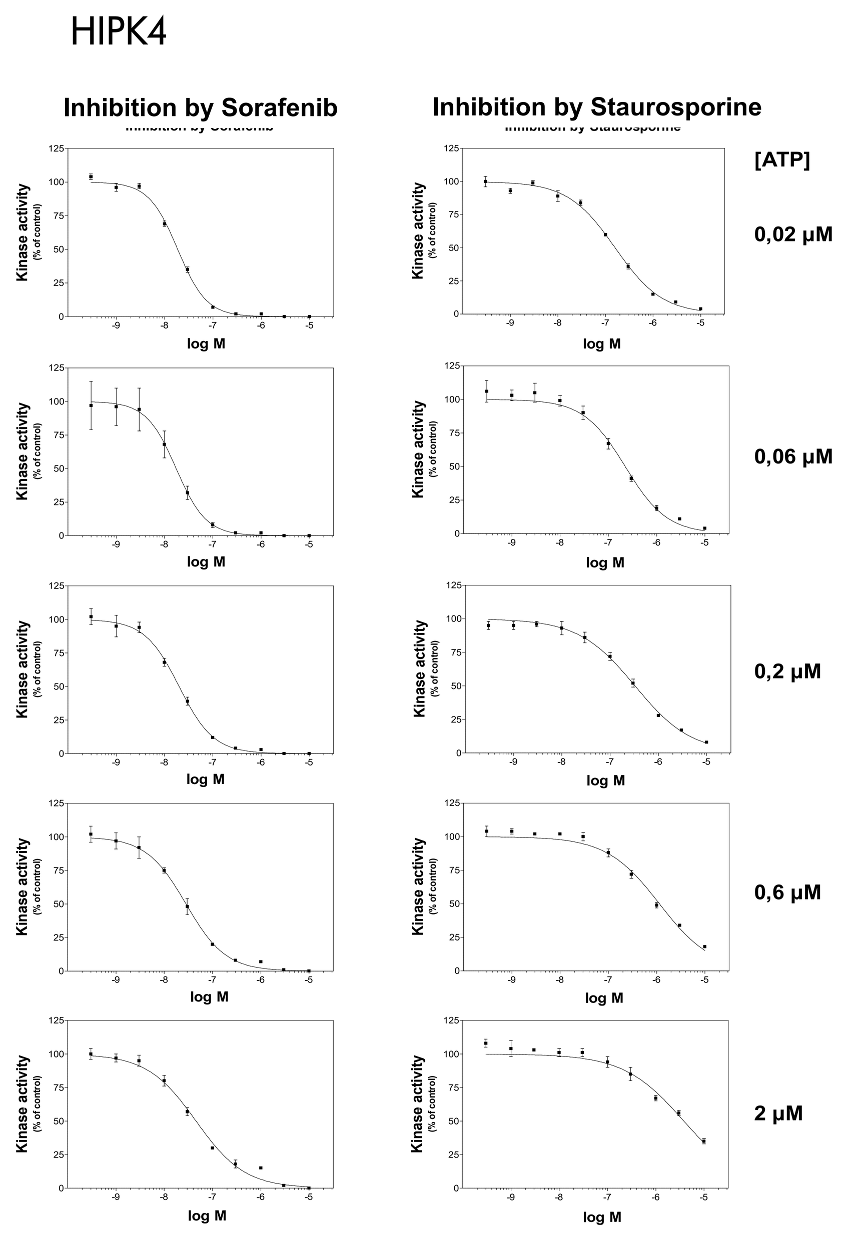
The assay is based on radiolabelled ^{33}P-g-ATP. Kinase and substrate are incubated in presence of ATP containing ^{33}P-g-ATP as tracer in 96 well plates. After stopping the kinase reaction, the reaction cocktail is transferred into 96 well filter plates and passed through the filter membrane by aspiration. Proteins are binding to the filter membrane and bound radioactivity is quantified by scintillation counting.

Assay Protocol:
in a 96 well polypropylene plate mix per well:
- 20 µl reaction buffer
- 10 µl substrate
- 10 µl kinase
- 5 µl compound
- Start reaction by addition of 5 µl ATP/^{33}P-g-ATP mixture
  Incubate 20 min at 30°C
- Stop reaction by addition of 20 µl H_3PO_4
- transfer reaction to 96 well filterplate
- aspirate reaction
- wash 3x with 200 µl 150 mM H_3PO_4
- add 50 µl scintillation cocktail
- determine cpm

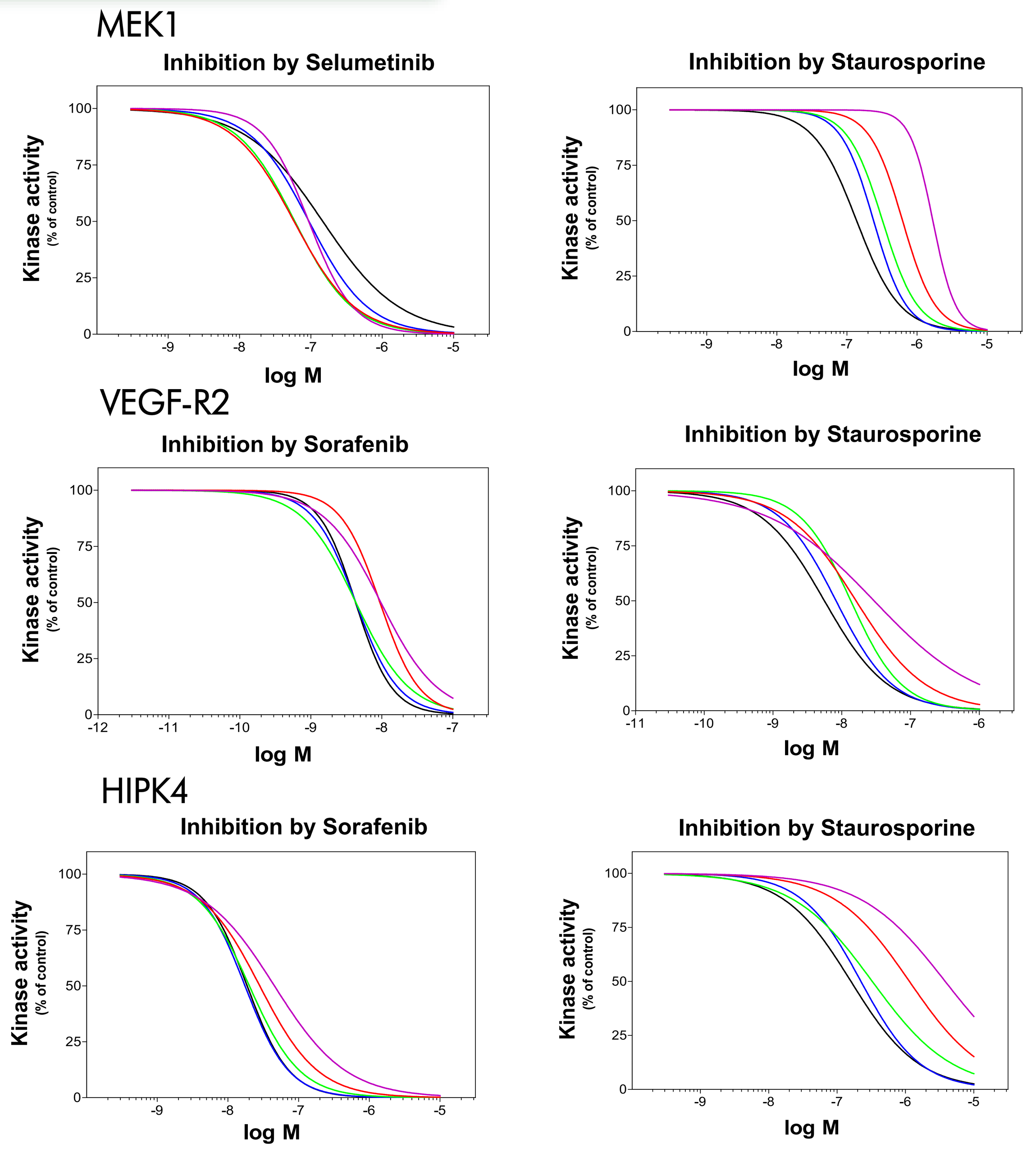
Final assay conditions:
70 mM HEPES pH 7.5
3 mM $MgCl_2$
3 mM $MnCl_2$
3 µM Na-orthovanadate
1.2 mM DTT
1 % DMSO
ATP*, substrate and kinase at variable concentration

*: [ATP]: 0.1 to 10x apparent K_M[ATP] of the respective kinase

Results



IC_{50} inhibition curves of HIPK4 for Sorafenib and Staurosporine.
Example for primary data and resulting dose-response curves.
HIPK4 K_M[ATP] apparent: 0.2 µM
each inhibitor concentration was determined at n=4



Inhibition curves of three kinases and two inhibitors at ATP concentrations ranging from $0.1x K_M$-ATP to $10x K_M$-ATP.
Dose response curves for all tested ATP concentrations for the respeictive kinases and inhibitors were plotted into one graph to visualize the influence of increasing ATP concentration on the IC_{50}.

	MEK1				VEGF-R2				HIPK4			
	Selumetinib		Staurosporine		Sorafenib		Staurosporine		Sorafenib		Staurosporine	
x ATP-K_M (µM)	IC_{50} (M)	Hill Slope	IC_{50} (M)	Hill Slope	IC_{50} (M)	Hill Slope	IC_{50} (M)	Hill Slope	IC_{50} (M)	Hill Slope	IC_{50} (M)	Hill Slope
0.1	1.50E-07	-0.81	1.40E-07	-1.40	4.30E-09	-1.70	5.60E-09	-0.94	1.90E-08	-1.40	1.60E-07	-0.88
0.3	9.40E-08	-1.10	2.40E-07	-1.90	4.30E-09	-1.40	8.20E-09	-1.10	1.70E-08	-1.40	2.20E-07	-1.00
1	5.90E-08	-2.00	3.20E-07	-1.70	4.30E-09	-1.10	1.30E-08	-1.20	2.00E-08	-1.20	3.30E-07	-0.74
3	5.80E-08	-1.00	6.20E-07	-1.90	9.40E-09	-1.60	1.60E-08	-0.86	2.80E-08	-1.10	1.10E-06	-0.79
10	9.30E-08	-1.40	1.70E-06	-2.70	9.70E-09	-1.10	2.90E-08	-0.56	4.60E-08	0.86	3.80E-06	-0.70
IC_{50}@10xATP-K_M / IC_{50}@0.1xATP-K_M	0.6		12.1		2.3		5.2		2.4		23.8	
Indicative for inhibition mode	non-ATP-competitive		ATP-competitive		mixed		mixed		mixed		ATP-competitive	

Numerical results of the experiments, including IC_{50} values and Hill-slopes for all tested kinases and inhibitors.
Ratios of the IC_{50} values at 10xATP-K_M and 0.1xATP-K_M are calculated. Based on the Cheng-Prusoff equation a prototypical ATP-competitive inhibitor is expected to exhibit a ratio of 10. Therefore the inhibition modes were classified as non-ATP competitive for a ratio of <2, as mixed for ratios of >2 and <10 and as ATP-competitive for ratios of >10.

Summary

- Non-ATP-competitive kinase inhibitors can be discriminated from ATP-competitive inhibitors by determining IC50 values in presence of increasing amounts of ATP
- The ATP-competitive or non-ATP-competitive characteristic of the same inhibitor (Staurosporine) may vary in a kinase dependent manner
- A type-2 inhibitor (Sorafenib) exhibited a mixed characteristic between ATP-competitive and non-ATP-competitive compounds

References

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