A high-throughput capable method with the potential to identify allosteric inhibitors of protein kinase activation cascades

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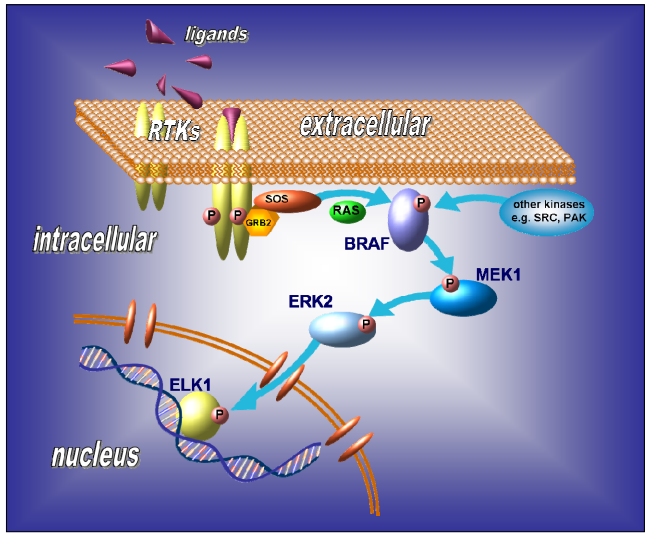
Introduction

Protein kinases are frequently found to be deregulated in cancer and are therefore important therapeutic targets. Hence the development of small molecule protein kinase inhibitors has been an ongoing task for more than ten years. There have been a number of successes and several compounds have been approved for medical application to date.

Almost all of these compounds exert their kinase inhibiting function by competing with ATP either by binding to the ATP-binding pocket directly or to conserved structures in close proximity, like the kinase activation loop, thereby stabilizing an inactive, non-ATP binding conformation of the kinase. Due to the identical enzymatic activity and conserved regulatory mechanisms across the whole kinome, the structural diversity of compounds which target the catalytic domain of kinases is limited. It has become evident that in order to obtain a higher specificity for small molecule kinase inhibitors they have to be developed to target allosteric sites apart from the core kinase domain.

Most of the high throughput capable kinase screening assays however have been developed to test the inhibitory effects of compounds against one kinase/substrate pair. Since the kinase has usually either to be active for in-vitro activity assays or able to bind ATP or ATP-analogues for binding/displacement assays, compounds which interfere allosterically with the activation mechanism rendering the kinase capable of binding/utilizing ATP in the first place are hard to detect by these approaches. The identification of such inhibitors requires an experimental setup in which the kinase is converted from its non-active to active conformation by its physiological upstream activator in absence or presence of the test compounds. Such assays may even address the effect of compounds on a complete signal transduction cascade including two or more upstream-downstream kinase activation pairs.

Schematic overview of the RAF-MEK-ERK pathway

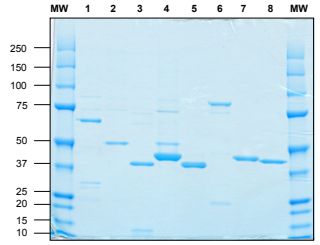


BRAF is activated by a multistep process, integrating input from multiple sources like activated receptor tyrosine kinases (RTKs) and other signal transduction kinases. Once activated by phosphorylation/dephosphorylation of certain amino acids, BRAF phosphorylates and activates MEK1 which in turn phosphorylates and activates ERK2. Multiple proteins are controlled by ERK2 dependent phosphorylation including ELK1, a transcription factor which is activated by ERK2.

Results

1. Setup of a HTS-capable MEK1-ERK2 in-vitro activation assay

A: Recombinant proteins



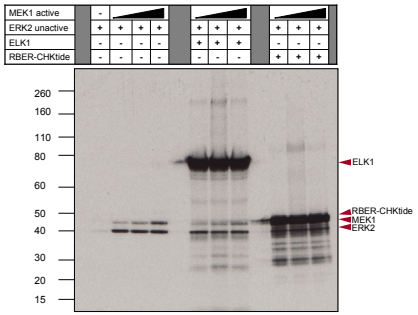
Purified proteins used for the individual assay experiments and cascade assay experiments.

Kinase and substrate components of the BRAF-MEK1-ERK2 signal transduction cascade were recombinantly expressed in insect cell (IC) or E.coli (EC), purified by affinity chromatography and either activated by in-vitro phosphorylation (A) or left untreated (U). Affinity tags were cleaved from a number of the proteins (C). Coomassie stained SDS-PA gel with 1 µg protein per lane/protein.

1: **BRAF** (IC, U, 69 kDa)
2: **MEK1** (IC, U, 48 kDa)
3: **ERK2** (EC, U, C, 42 kDa)
4: **MEK1** (IC, A, C, 44 kDa)
5: **ERK2** (EC, A, C, 42 kDa)
6: **ELK1** (IC, C, 73 kDa)
7: **MEK1 K97M** (IC, C, 44 kDa)
8: **ERK2 K54R** (EC, 45 kDa)

MW: molecular weight marker, (Bio-Rad Precision broad range, prestained)

B: in-vitro phosphorylation of MEK1-ERK2 cascade components



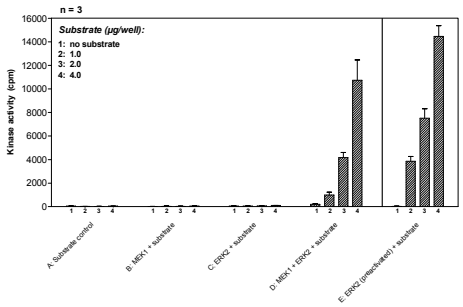
Autoradiogram of the in-vitro MEK1-ERK2 activation assay.

Recombinant kinases MEK1 (pre-activated by BRAF), ERK2 (non-activated) and substrate proteins ELK1 and RBER-CHKtide were incubated in the combinations described for 60 mins at 30°C in presence of 3 µM ATP and approx. 9*10⁶ cpm of ^{33}P-γ-ATP. Amounts per reaction were 25, 50, 100 ng MEK1, 500 ng ERK2 and 5 µg of ELK1 and RBER-CHKtide respectively.

No phosphorylation of ELK1 and RBER-CHKtide was detectable with ERK2 in absence of MEK1 (data not shown)

C: Transfer of the MEK1-ERK2 activation cascade to a HTS-capable assay format

Assay technology: Flashplate® based radiometric kinase activity assay



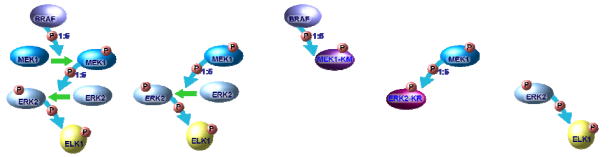
Flashplate® based MEK1-ERK2 activation assay

Non-activated ERK2 (50 ng/well) was incubated with active MEK1 (5 ng/well) in presence of ERK2 substrate RBER-CHKtide (0/1/2/4 µg/well) at an ATP concentration of 5 µM. Pipetting sequence was ERK2 ± compound – 10 min incubation at RT (compound binding) – MEK1+RBER-CHKtide+ATP/ ^{33}P-γ-ATP. The reactions were incubated at 30°C for 60 min and protein bound radioactivity determined by scintillation-counting. MEK1 (active) (B) or ERK2 (non-activated) (C) alone did not increase the signal compared to the substrate control (A). In presence of catalytic amounts of MEK1 ERK2 becomes activated (D) and phosphorylates the substrate comparably to pre-activated ERK2 (E).

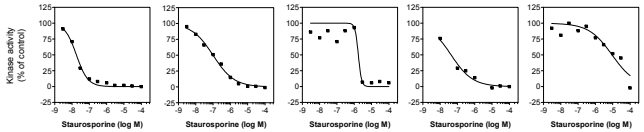
In a case study using a library of >1500 kinase focused compounds a hit rate of 10.1% could be achieved using this experimental setup. Several inhibitors of the MEK1-ERK2 activation cascade were further analysed by comparing their inhibitory activity against the cascade with their effect on the individual kinase/substrate pairs. 7.5% of the compounds showed an inhibitory effect in the activation assay but not in the individual assays, indicating a potential allosteric mode of action e.g. by interfering with binding of MEK1 to unactivated ERK2 or masking of activatory phosphorylation sites.

2: BRAF-MEK1-ERK2 – cascade vs. individual assays

A three step BRAF-MEK1-ERK2 assay was established using the radiometric Flashplate® based assay technology. Kinases were used in a 1:5:5 ratio with ELK1 as final substrate for ERK2. No phosphorylation of ELK1 by BRAF or MEK1 could be detected. In this cascade assay setting IC_{50} values for several inhibitors were determined and compared to the IC_{50} values obtained using each kinase/substrate separately.

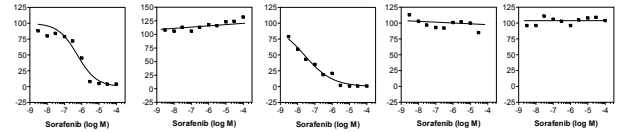


A: Staurosporine



IC_{50}	20 nM	90 nM	1700 nM	43 nM	9100 nM

B: Sorafenib



IC_{50}	560 nM	n/a	24 nM	n/a	n/a

Depending on the location and number of points of inhibition inside the activation cascade a compounds potency readout may appear higher or lower than when determined for the individual kinase/substrate pairs. While an inhibitor acting early in the cascade may appear less potent due to signal enhancing properties of the subsequent cascade components than if tested isolated on its main target, an allosteric inhibitor could appear more potent in the cascade than in the individual assay due to the absence of its actual target mechanism in the individual assay (e.g. not yet activated kinase).

Summary

- recombinant kinases belonging to the RAF-MEK-ERK pathway have been purified in different activation states (non-activated, activated, inactive)
- a two step in-vitro activation assay for MEK1-ERK2 has been established to screen >1500 compounds. A significant number of compounds inhibited the MEK1-ERK2 cascade differentially as compared to the isolated MEK1 or ERK2 activity assays indicating a potential allosteric mode of action
- a three step BRAF-MEK1-ERK2 assay was established which could potentially be used to identify inhibitors acting by mechanisms appart from directly interfering with kinase activity

Conclusion

Screening compounds against a signalling cascade instead of single kinase/substrate pairs results in a more comprehensive overall picture of a compound's inhibitory potential. It may also detect inhibitors with modes of action apart from directly interfering with the enzymatic activity of a kinase, e.g. substrate competition or masking activatory phosphorylation sites.