# Competitive Assay Approach: Binding of Small Molecules to the Active Form of p38

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## Abstract

**p38α is considered as the key isoform involved in modulating inflammatory response in rheumatoid arthritis and inflammatory pain and is therefore a key target for compound screening in pharmaceutical industry. In this study we demonstrate the use of MicroScale Thermophoresis to perform an essentially label-free measurement of small molecule binding to protein targets by using a competitive approach. This approach therefore allows screening projects aiming to identify and characterize site-specific binders or binders allosterically influencing a specific binding site out of a compound library. Furthermore this approach also can be used to screen for compounds that interrupt protein-protein, protein-peptide or protein-nucleic acid interactions.**

## Introduction

p38 is a serine/threonine protein kinase in the mitogen-activated protein kinase (MAPK) family. There are four isoforms of p38 (p38α, p38β, p38γ, and p38δ), and p38α is considered as the key isoform involved in modulating inflammatory response in rheumatoid arthritis and inflammatory pain (Dominuez *et al.* 2005) Two well characterized small molecule antagonists SB203580 (Davies *et al.*, 2000) and the clinical candidate BIRB-796 (Lee and Dominguez 2005) were used in this study. Whereas SB203580 competes with ATP for the binding site on the kinase, BIRB-796 binds adjacent to the active site in a new allosteric binding pocket and indirectly inhibits enzymatic activity by affecting the conformation of the ATP site (Pargellis *et al.* 2002).

Our experiment describes the competition of a fluorescent tracer molecule (ATP mimetic tracer199, Invitrogen) bound to the MAP kinase p38 by non-labeled small molecules (SB203580, BIRB-796).

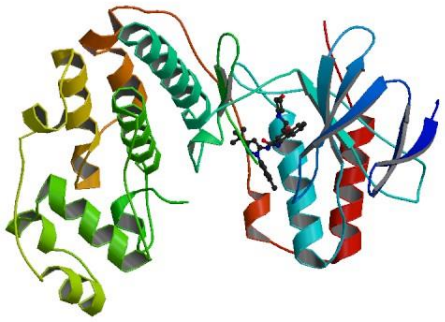

**Fig. 1** Crystal structure of the p38-BIRB-796 complex

The competition approach allows for a basically label-free measurement of the interaction between a compound and a protein since all molecules of interest are not labeled. Fig. 2 shows the principle of this approach. A complex of a protein and a fluorescently labeled molecule (grey with red star) and a protein is formed. This complex is mixed with a serial dilution of a small molecule of interest (blue). When the small molecule is binding the fluorescently marked tracer is set free, generating a strong MST signal (i.e. the difference of protein/tracer thermophoresis to thermophoresis of free tracer).

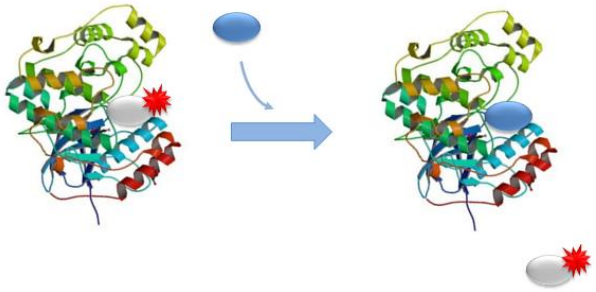

**Fig. 2** Competitive approach using a fluorescently-labeled tracer (grey with red star). Adding unlabeled compounds (blue) that compete for the same binding site or allosterically induce conformational changes of the tracer binding site will lead to competive removal of the tracer ending up with free labeled tracer.

The affinity of the non-labeled small molecule to the unlabeled protein can be determined precisely by using the equation

$$K_i = [I]_{50} / ([L]_{50} / K_d + [P]_0 / K_d + 1)$$

In this equation $K_d$ is the dissociation coefficient of the tracer and protein, $P_0$ is the concentration of the protein at 0 % binding, $I_{50}$ is the concentration of free ligand at 50 % binding, $L_{50}$ the concentration of the free tracer at 50 % binding. $K_i$ is the affinity of the non-labeled small molecule for the unlabeled protein.
Please note, this type of assay can also be performed with a labeled peptide, nucleic acid or even a protein. By doing so it is not only possible to analyze if a molecule is binding, but also to infer its activity (e.g. if a compound is able interrupting a protein-protein interaction).

## Results

First the binding of tracer199 to the active form of the kinase p38α was evaluated. The results are shown in Fig. 3.

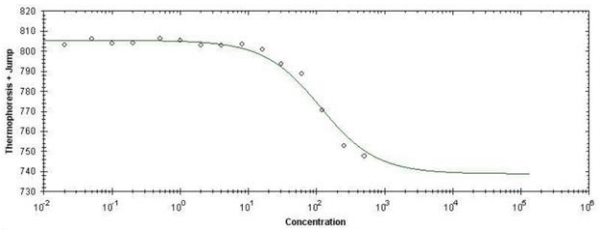


**Fig. 3** Binding of tracer199 to p38α

It shows a decreasing MST signal with increasing p38 concentration (starting at 805 units, decreasing to 738 units) and a sigmoidal behavior that allows deducing a $K_d$ of about 80 nM.

With the experiment shown above, the tracer-protein system is well characterized and a competition experiment can be performed. A prepared stock solution of the tracer199-p38 complex (see Materials and Methods) was mixed with a serial dilution of the small molecule SB203580 (MW = 377.4 Da) starting at 4 μM (see Fig. 4). This molecule is known to have a high affinity to the protein p38 $IC_{50}$ = 34 nM *in vitro* and 600 nM in cells.

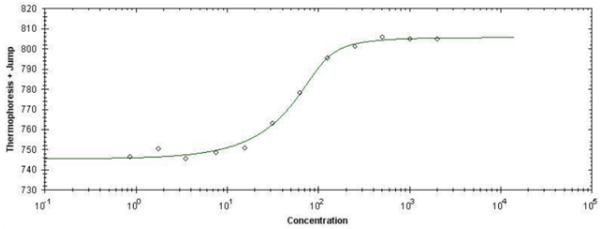


**Fig. 4** Competitive binding of SB203580 to p38α loaded with fluorescently marked tracer199

The signal starts at about 748 units which indicates that most of the tracer is in complex with the protein. When increasing the concentration of SB203580, the MST signal increases to about 805 units, which is exactly the signal level we expect for free tracer199 thermophoresis. The signal allows to determine an $IC_{50}$ of 100 nM and thus a $K_d$ of 26 nM (according to equation 1) in good accordance with literature values (Davies *et al.* 2000).

## Conclusion

This competitive approach using a labeled molecule that is replaced allows measuring a dissociation constant at essentially label-free conditions. It also enables a site specific detection of the binding since a MST signal of the respective amplitude is only observed when the titrated small molecule binds to the same site as the tracer or allosterically influences this specific binding site. In addition to these advantages, this approach also allows to setup larger screening projects on the Monolith instrument: In case the protein is labeled and no tracer is used, the signal direction and amplitude will differ depending on the chemistry of the small molecule, its binding site and the conformational changes induced. This is different when a tracer is used. The MST signal direction (increasing or decreasing) is independent of the small molecule and its binding mode/site. The amplitude only depends on the amount of tracer that has been released by the compound. This allows screening for binders by using just few different concentrations and defining a certain amplitude cut-off value that defines which molecules are interesting for more detailed

analysis. Furthermore this approach also can be used to screen for compounds that interrupt protein-protein, protein-peptide or protein-nucleic acid interactions.

## Material and Methods

**Assay buffer**
For the experiment described here, a 50 mM Tris buffer pH 7.6 containing 150 mM NaCl, 10 mM $MgCl_2$ and 0.05 % Tween-20 has been used.

**Instrumentation**
The measurements were done on a Nanotemper Monolith NT.115 instrument.

For evaluation of the tracer199 (Invitrogen) binding to p38 10 µl of a 50 nM stock solution of tracer199 were mixed with 10 µl of a serial dilution of p38 starting at 1 µM. The samples were incubated for 10 minutes and measured at an MST power of 15 % and a LED power of 50 % with a laser-on time of 30 seconds and a laser-off time of 5 seconds.
For the competition experiment a stock solution of 150 nM p38 and 25 nM tracer199 was prepared. In this concentration range a sufficient amount of the fluorescent tracer is bound to the protein. Please note that not all tracer molecules have to be in complex with the protein. However, free tracer will decrease the amplitude of the MST signal. 10 µl of the stock solution of the tracer199-p38 complex was mixed with a serial dilution of the small molecule SB203580 starting at 4 µM and measured under the same conditions as before.

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