Automated Fragment Screening using MST

Application Note NT021

Case Study on Automated Screening Project of a Fragment Library against MEK1 kinase

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Abstract

Biophysical approaches are an integral part of the drug discovery process to asses binding constants of molecular interactions, which are in turn required for efficient lead optimization. Here we show that MicroScale Thermophoresis (MST) is a powerful technique to rapidly determine binding constants in fragment screening approaches.

Introduction

MST detects the directed movement of fluorescent molecules in microscopic temperature gradients (Jerabek-Willemsen et al, 2011). Small changes in the hydration shell of these molecules, caused for instance by binding of small molecules, results in changes in their thermophoretic movement which can be used to derive binding constants. MST is a highly sensitive method that can detect fluorophores at low picomolar concentrations and requires only a few µl of sample material.
Employing the newly developed Monolith NT.Automated instrument, a library containing 193 pre-selected fragments was screened for interactions with the drug target mitogen-activated protein kinase 1 (MEK1).

Aberrant signaling in the pathway is associated with unregulated cell growth, and targeting this cascade has become a viable means of developing anticancer therapies (Saini et al, 2013; Salama & Kim, 2013). MEK1 is a key component of this pathway and is responsible for the phosphorylation and activation of downstream ERK proteins.

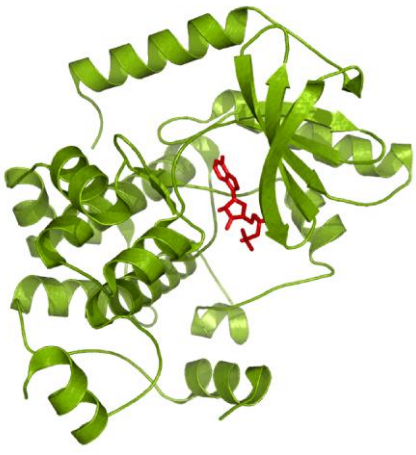

Figure 1: Crystal structure of the MEK1 kinase (green) with a bound nucleotide (red stick model) in the active site (PDB 3ZLX).

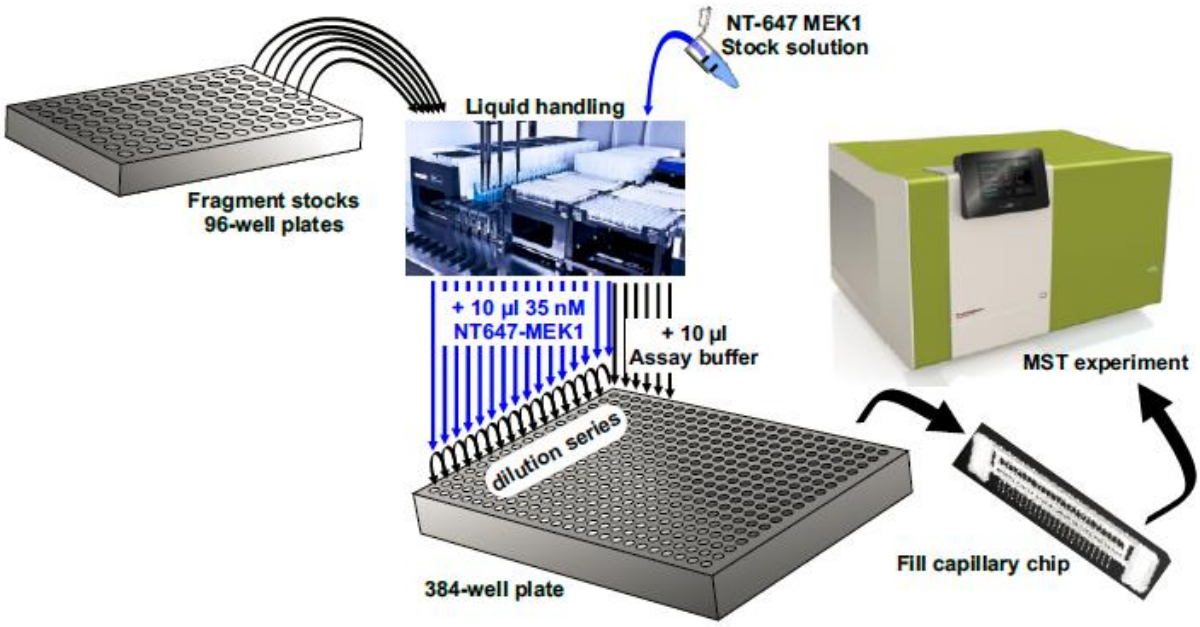

Figure 2: MST Screening Setup. Fragment dilution series and addition of fluorescently labeled MEK1 is carried out by a liquid handling device in standard microtiter plates. After filling capillary chips with the reaction mixture containing NT-647-MEK1 and diluted fragment, MST is monitored in the Monolith NT.Automated.

Results

Using the Monolith NT.Automated instrument, we screened a library containing 193 pre-selected fragments for their interaction with the drug target MEK1 (Figure 2). The same library has been previously used in a combinatory approach employing differential scanning fluorimetry (DSF) and surface plasmon resonance (SPR) (Amaning et al, 2013). The aim of our project was to use MST to derive binding constants for the MEK1-fragment interactions, and to compare the MST ranking results with those obtained from other biophysical methods.

To establish the assay, we first analyzed the interaction of NT-647 fluorescently labeled MEK1 with its natural substrate ATP, which later served as a positive control. A total of 13 ATP control experiments were performed throughout the screening to ensure MEK1 functionality. The average dissociation constant K_d of these control experiments was 9.4 +- 0.8 µM, corroborating the robustness of the screening approach.

Analyzing the binding of all 193 fragments, we identified and ranked > 70 binders according to their affinities, with K_ds ranging from the low µM to low mM range. 16 fragments displayed K_ds below 100 µM. Importantly, 7 out of 8 crystal-structure validated hits were among the top-fifteen fragments from our MST-ranking (Figure 3).

Interestingly the MST ranking showed a very strong correlation with a qualitative DSF screening (Figure 4), while a SPR screening failed to identify positive hits and showed no correlation with the DSF ranking (data not shown). Instead, SPR picked up several false-positives, which were easily identified by MST due to their protein aggregating/denaturating effects (Figure 5).

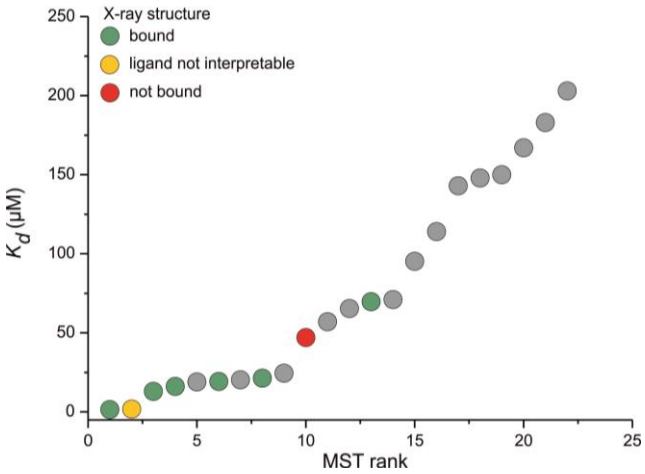

Figure 3: 7 out of 8 crystal-structure-validated hits (green and yellow dots) were among the top-fifteen fragments from the MST-ranking.

These effects can either be identified by irregular “bumpy” MST traces or loss of fluorescence due to protein degradation. Thus, MST can identify false-positive binding events caused by protein aggregation/denaturation, which can be missed by other methods. Moreover, MST robustly detects the binding of small fragments with molecular weights in the range of 150-300 Da, which cannot be detected by SPR.

Taken together, high-throughput binding analysis by MST is a valuable tool that complements qualitative screening methods and circumvents false-positive results which are commonly picked up by other biophysical screening methods.

In addition to the known advantages of MST such as little sample consumption, easy handling, rapid assay optimization and straight-forward data analysis, MST moreover provides information about protein quality, different binding sites, protein stability and aggregation. Moreover, competition assays can be applied on the same platform to verify binding sites e.g. by competing with ATP in the binding pocket.

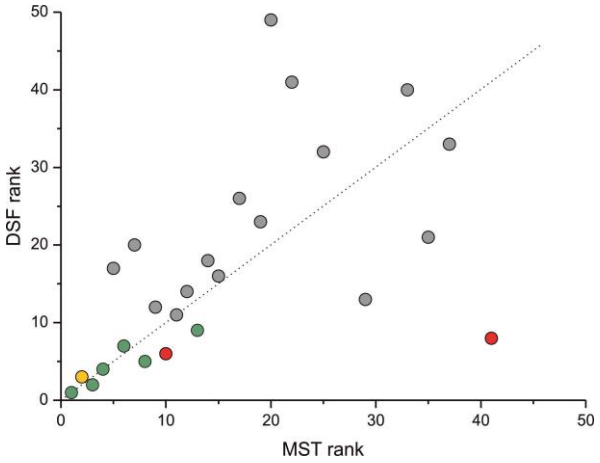

Figure 4: Quantitative MST ranking showed a clear correlation with a qualitative DSF screen

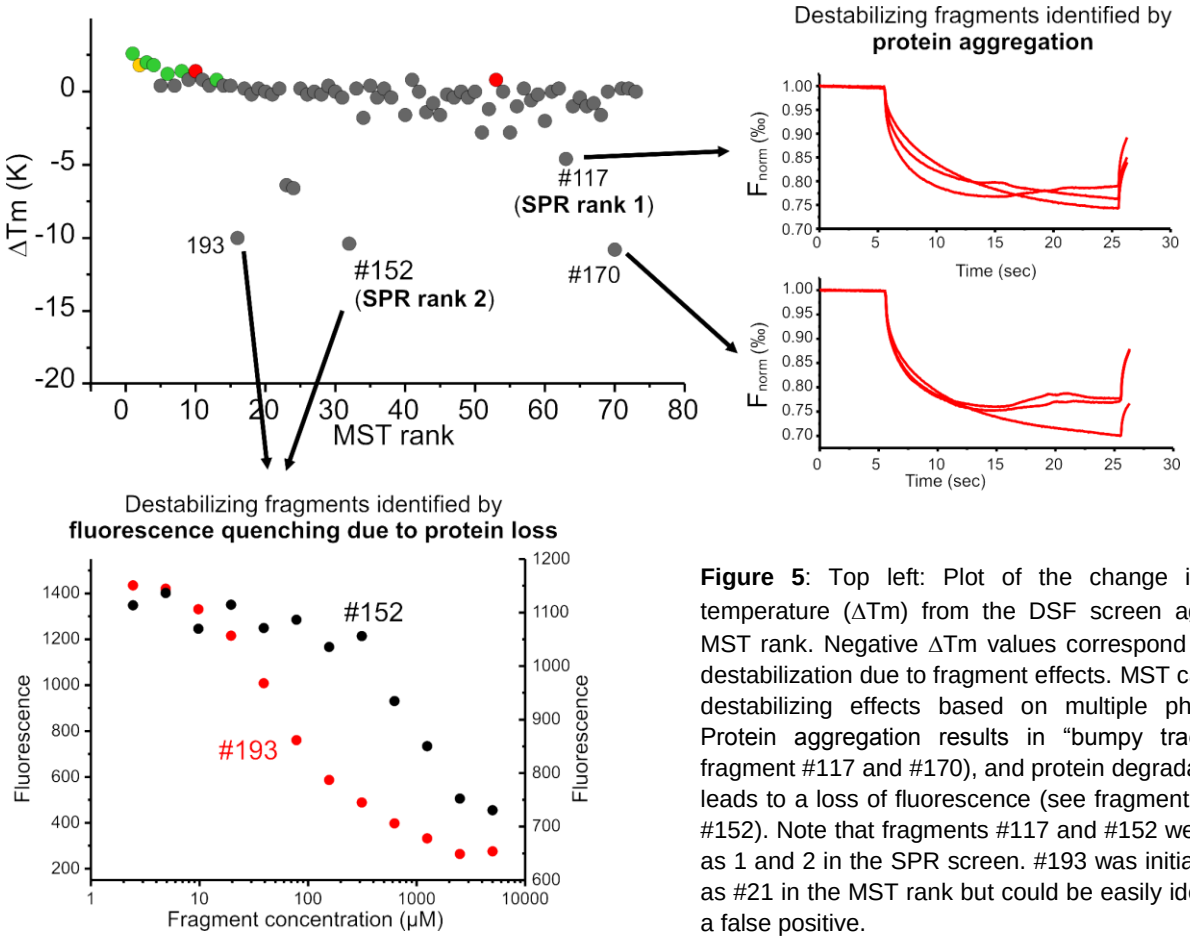

Figure 5: Top left: Plot of the change in melting temperature (ΔTm) from the DSF screen against the MST rank. Negative ΔTm values correspond to protein destabilization due to fragment effects. MST can identify destabilizing effects based on multiple phenomena: Protein aggregation results in “bumpy traces” (see fragment #117 and #170), and protein degradation often leads to a loss of fluorescence (see fragment #193 and #152). Note that fragments #117 and #152 were ranked as 1 and 2 in the SPR screen. #193 was initially ranked as #21 in the MST rank but could be easily identified as a false positive.

Conclusion

The Monolith NT.Automated allows for screening libraries of compounds or fragments in a timely manner with very low sample consumption. In addition, the novel capillary chip format allows for easy handling and the integration of MST experiments into a fully automated setting.
Notably, in the case study presented here 7 out of 8 crystal-structure validated hits were among the top-fifteen fragments from our MST-ranking, whereas false-positive hits from orthogonal techniques could be identified directly from the screening results. Additionally, competition assays can provide further insights into the binding mechanism of drug candidates.
Therefore, high-throughput MST analysis is a perfect asset in modern drug discovery processes.

Material and Methods

Protein construct and modification.
The protein construct of MEK1 (residues 37-383) was purchased from Crelux (www.crelux.com). MEK1 was fluorescently labelled on lysine residues with NT-647 fluorophores using the Monolith NT.115 Protein Labeling Kit RED-NHS, resulting in an average labeling degree of 1.1 +/- 0.2. NT-647 MEK1 was eluted in assay buffer (50 mM Tris pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT) and frozen at -80 °C in 8 µl aliquots. The total amount of protein used for the screening was 40 µg.

Assay development and optimization.
After thawing, NT-647 MEK1 was centrifuged for 10 minutes at 22000 x *g* to remove protein aggregates. Subsequently, NT-647 MEK1 was diluted to 30 nM in assay buffer, filled into NT.115 standard capillaries and analyzed in a NT.115^{Pico} device. The capillary scan revealed adsorption of the labeled molecule to capillary walls. Addition of either Tween-20 (0.05 %) or Pluronic F 127 (0.1 %) (both from Sigma Aldrich) prevented protein adsorption in standard capillaries. All subsequent experiments were carried out in NT.115 standard capillaries and Assay buffer containing Pluronic F-127 (0.1 %).
As a positive control, we analyzed the binding of ATP and ADP to NT-647 MEK1, yielding K_ds of 9.1 +- 0.5 µM and 7.5 +- 0.2. In the absence of $MgCl_2$, the K_d of the ATP-MEK1 interaction increased to > 140 µM, which is in line with the previously reported Mg^{2+}-dependence of MEK1 (Fischmann et al, 2009; Sheth et al, 2011; VanScyoc et al, 2008).
The fragment library was provided in DMSO in 96-well plates. Each well contained 5 µl aliquots with a fragment concentration of 100 mM in DMSO. The compounds were pre-diluted in Assay buffer to reach a concentration of 10 mM. Next, a 1:1 dilution series in Fragment buffer (50 mM Tris pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 0.1 % Pluronic F-127, 10 % DMSO) was prepared (10 µl final volume) in 384-well plates (non-binding, Greiner Bio One) using a Hamilton Microlab Star^{LET} liquid handling system. 10 µl of a 70 nM NT-647-MEK1 solution was added to each fragment dilution, mixed, loaded into NT.standard capillaries and subject to MST analysis.

Data analysis
The dissociation constant (K_d) of fragment-MEK1 interactions was quantified by analyzing the T-Jump signal at a MST power of 80 % using the NT.Analysis software. Fragments were ranked according to their affinity (highest first). Fragments displaying abnormal MST traces or binding amplitudes with less than 3 counts were excluded from the analysis.

References

Amaning K, Lowinski M, Vallee F, Steier V, Marcireau C, Ugolini A, Delorme C, Foucalt F, McCort G, Derimay N, Andouche C, Vougier S, Llopart S, Halland N, Rak A (2013) The use of virtual screening and differential scanning fluorimetry for the rapid identification of fragments active against MEK1. *Bioorganic & medicinal chemistry letters* **23:** 3620-3626

Fischmann TO, Smith CK, Mayhood TW, Myers JE, Reichert P, Mannarino A, Carr D, Zhu H, Wong J, Yang RS, Le HV, Madison VS (2009) Crystal structures of MEK1 binary and ternary complexes with nucleotides and inhibitors. *Biochemistry* **48:** 2661-2674

Jerabek-Willemsen M, Wienken CJ, Braun D, Baaske P, Duhr S (2011) Molecular interaction studies using microscale thermophoresis. *Assay and drug development technologies* **9:** 342-353

Saini KS, Loi S, de Azambuja E, Metzger-Filho O, Saini ML, Ignatiadis M, Dancey JE, Piccart-Gebhart MJ (2013) Targeting the PI3K/AKT/mTOR and Raf/MEK/ERK pathways in the treatment of breast cancer. *Cancer treatment reviews* **39:** 935-946

Salama AK, Kim KB (2013) MEK inhibition in the treatment of advanced melanoma. *Current oncology reports* **15:** 473-482

Sheth PR, Liu Y, Hesson T, Zhao J, Vilenchik L, Liu YH, Mayhood TW, Le HV (2011) Fully activated MEK1 exhibits compromised affinity for binding of allosteric inhibitors U0126 and PD0325901. *Biochemistry* **50:** 7964-7976

VanScyoc WS, Holdgate GA, Sullivan JE, Ward WH (2008) Enzyme kinetics and binding studies on inhibitors of MEK protein kinase. *Biochemistry* **47:** 5017-5027