Spectral Shift & MST-TRIC: New Biophysical Technologies Supporting Kinase Drug Discovery Programs

M. Sigoillot[1], C. Bornet[1], A. Baron[1], R. Merceron[2], T. Annaval[2], P. Adams[2], V. Siso[1], P. Bourdilleau[1], C. Gomes Correia[1], C. Legros[1], V. Porkolab[1]
1.Eurofins Discovery | Poitiers, France, 2.Eurofins DiscoverX® | Poitiers, France

Abstract

PIM3 kinase, which belongs to the Ca2+/calmodulin-dependent protein kinase (CaMK) group, exhibits serine/threonine kinase activity. Similar to other members of the PIM family (i.e. PIM1 and PIM2), PIM3 can prevent apoptosis, promote cell survival and protein translation. In addition, identifying potent and selective hits for PIM3 is of particular interest for the treatment of cancer.
Using Eurofins Discovery's state-of-the art hit finding platform, we designed different comprehensive screening cascades where biophysics is used for primary screening (fragment-based screening approach) and/or orthogonal assays, such as SPR, MST, TSA, ITC, etc.

Several biophysical strategies can be used for this step of a drug discovery program, and it is the target properties which drive the selection of the most suitable technology. The novel Spectral Shift technology, using the Dianthus (well-known for the MST), was the most appropriate method for the PIM3 drug-discovery program.

Methods

Protein production. Cter His-tagged PIM3 was produced in insect cells from 10L batches and purified by IMAC and gel filtration into 50 mM Hepes/NaOH pH 7.5, 150 mM NaCl, 5 % glycerol, 1 mM TCEP.

Spectral Shift experiments. PIM3 was labeled using the His-Tag Labeling Kit RED-tris-NTA 2nd generation (ref: NT-L118, Nanotemper) following supplier instructions in PBS-P+. Compounds were dispensed by Echo acoustic technology. For primary screen, 250 nL of fragments at 150 µM final concentration were tested in duplicate with 50 nM PIM3 in PBS-P+ (1% final DMSO). For dose-response experiments, two-fold dilutions series were prepared at 30 µM or 600 µM for compounds or fragments, respectively.

Thermal Shift Assays. Unfolding profiles of PIM3 were recorded using CFX384 Touch (BIORAD). 5 µL of PIM3 at 4 µM and 20X SyproOrange were mixed with 5 µL of compounds in PBS, 1% DMSO. Measurements were conducted in triplicates.

FBS Cascade: Spectral Shift for PIM3 Primary Screen

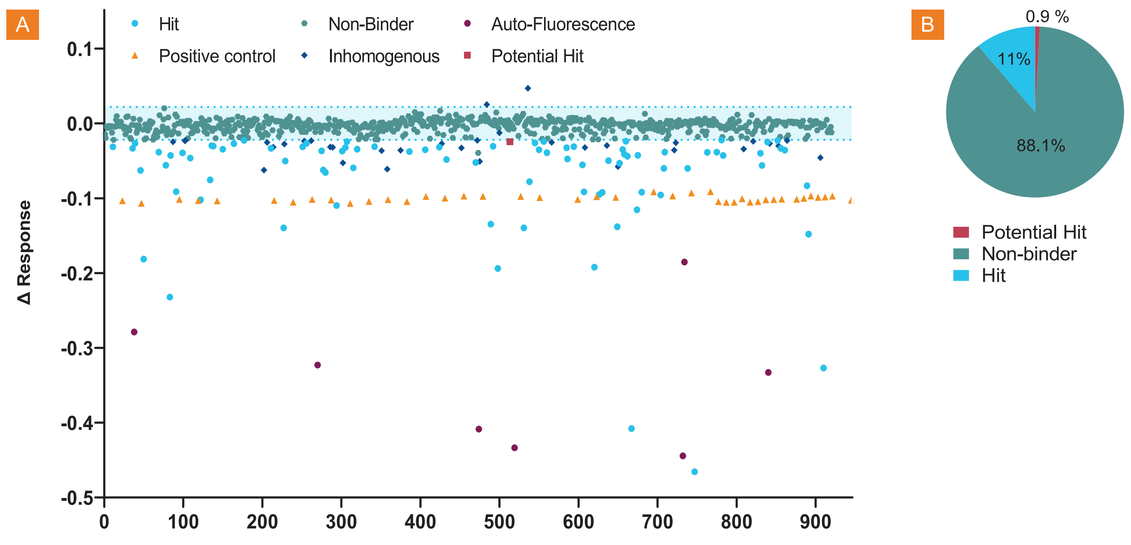

Figure 4. Screening of PIM3 binders by Spectral Shift Technology. A. A selection of 826 fragments were tested at 600 µM final concentration, in duplicates. Hits are shown as light blue circles. ATPγS was used as positive control at 100 µM and depicted as orange triangles. B. Pie chart summarizing the proportion of binders after primary screen (11%). Potential binders include potential hit, inhomogeneous and auto-fluorescence signals.

Spectral Shift Technology

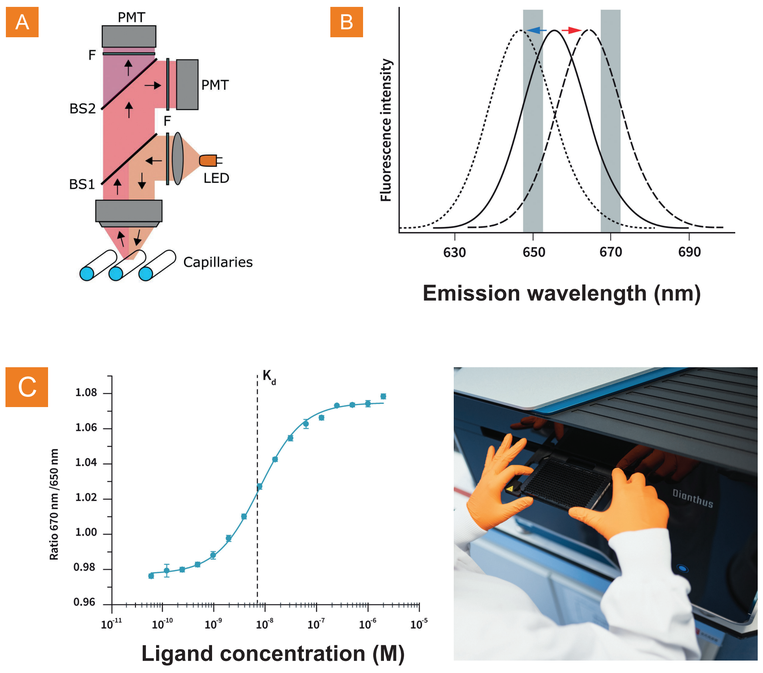

Figure 1. Experimental epifluorescence setup and Spectral Shift signal. A. An amber LED produces excitation light of a peak wavelength of 592 nm that matches the secondary, shorter absorption peak of a near-infrared fluorophore. The light is reflected on a beamsplitter (BS1, 615 nm) and excites fluorescence within a capillary.[3] The red-shifted emission then passes BS1 and is divided by a second beam splitter (BS2, 660 nm) into a lower and higher wavelength component. Filters (F) further clean up the emission light before being collected in photon-multiplier-tubes (PMTs). B. Red dyes lead to a decrease of the 650 nm and an increase of the 670 nm fluorescence or vice versa.[3] C. Plotting the 670 nm/650 nm ratio against the logarithmic concentration of a non-fluorescent ligand leads to a sigmoidal binding curve that can be used to extract the K_D value of the interaction.[3]

FBS Cascade: Spectral Shift for PIM3 DRC Confirmation

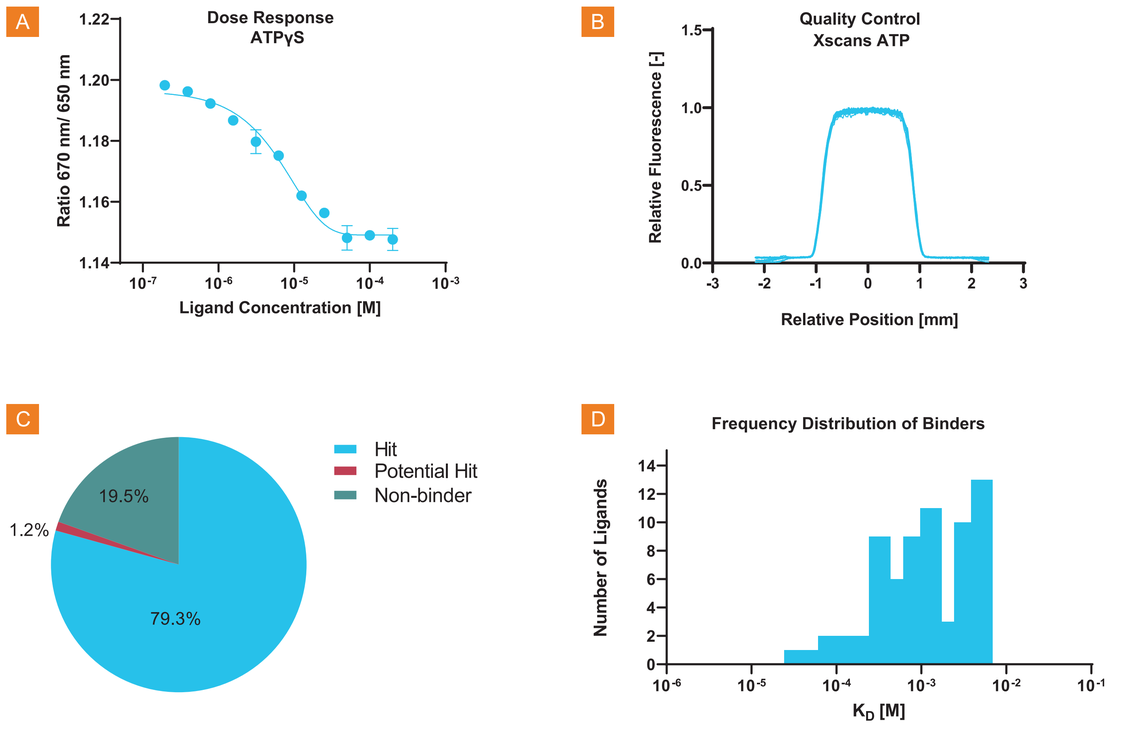

Figure 5. Positive control, QC and Hit Characterization using Spectral Shift. A. Example of dose response curve obtained with the positive control (ATPγS). B. X scan graphs used as quality control for well homogeneity and aggregation. C. Pie chart summarizing the proportion of confirmed binders after dose response titrations (79.3%). Fragments were prepared in two-fold dilutions series with a top concentration of 600 µM final concentration, in PBS-P+ 2% DMSO. D. Frequency distribution of binders ranked by binding affinities (K_D). The top 33 fragments were selected for TSA orthogonal assays.

PIM3 Screening Cascade Strategies

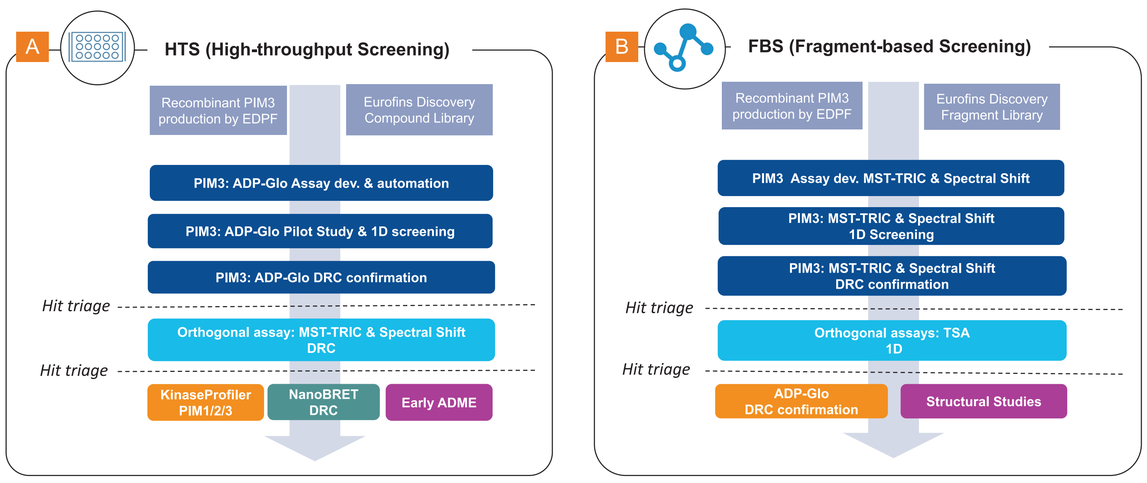

Figure 2. Examples of PIM3 screening strategies for Hit Identification. A. Starting from a subset of 71,000 compounds from a 600,000-compound library, 140 active hits using the ADP-Glo™ PIM3 activity assay were identified. "Active" hits were then confirmed using MST-TRIC & Spectral Shift in order to measure the direct interaction between compounds and recombinant PIM3 designed by Eurofins DiscoverX. Ready-to-go selectivity (vs PIM1 and PIM2), NanoBRET™, early ADME assays, validate the most promising and selective series for PIM3. B. PIM3 fragment-based screening was performed with a subset of 826 fragments from a 3,133-fragment library using MST-TRIC & Spectral Shift approach. Fragment binding was confirmed by a DRC confirmation and orthogonal assays.

FBS Cascade: PIM3 Orthogonal Assays by Thermal Shift Assay (TSA)

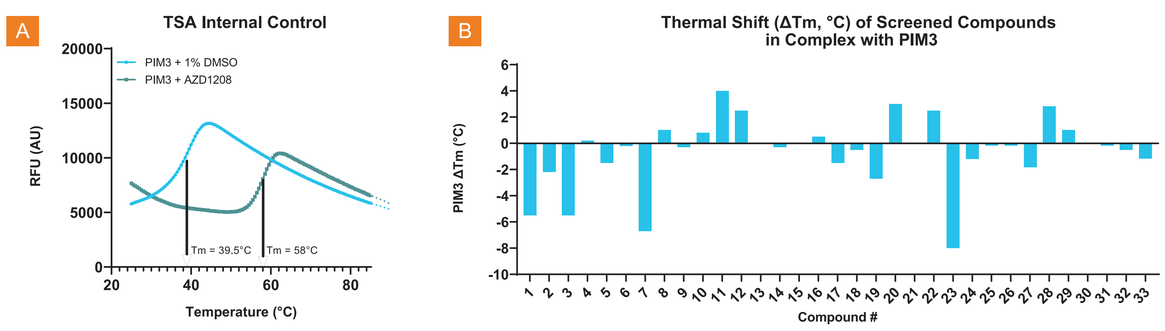

Figure 6. Thermal Shift Assay was used to assess the effect of fragments on PIM3 stability. A. Example of melting curves obtained using PIM3 apo-form (blue curve) and PIM3 in presence of AZD1208 inhibitor at 100 µM (green curve), used as positive control. AZD1208 leads to PIM3 thermal stabilization (+18.5°C). B. The 33 fragments identified by Spectral Shift were tested at 600 µM in triplicates. Fragments with ΔTm > 3 SD and variation >1°C were considered as binders and stabilizers.

HTS Cascade: Spectral Shift for PIM3 Orthogonal Assays

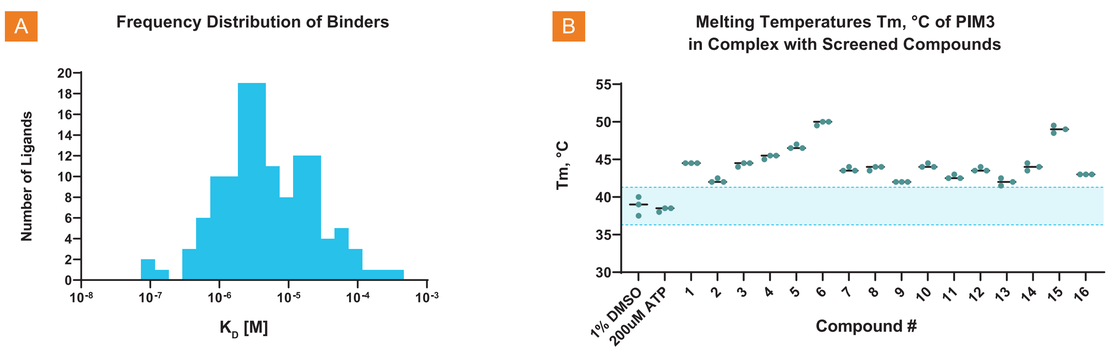

Figure 3. Biophysics for orthogonal assays and binding hit triage. A. Spectral Shift technology allowed ranking of compounds by affinity (K_D values). 140 compounds were tested in dose-responses and 128 binders were identified as binders for PIM3 kinase (92% Hits). 22 compounds showed subnanomolar-range affinities. B. Summary graph of PIM3 melting temperature in complex with the top 16 selected compounds. Compounds with ΔTm > 3 SD and variation >1°C were considered as binders. All compounds induce significant PIM3 thermal stabilization.

Summary

- Spectral Shift is a sensitive technology that enabled the rapid identification of hits from both small molecules and fragments in single dose experiments.
- It was important to implement an easy and rapid assay that would monitor the ability of PIM3 to bind natural ligand, compounds and fragments in solution. Using the Echo's acoustic droplet ejection, nanoliter dispensing in 384-well plates, which vastly reduces sample consumption, robust data can be generated quickly, driving the MedChem analysis for Hit triage.
- Hit confirmation can be further investigated by dose-response experiments and allows for the ranking of compounds by affinity (K_D determination). TSA is used as a complementary biophysical assay enabling the identification of PIM3 stabilizers and destabilizers.
- The 16 hits selected based on MedChem analysis are currently in a Hit-to-Lead program.

References

3. Langer A., Ludecke A., Bartoschik T., Cehlar O., Duhr S., Baaske P. Streicher W. A New Spectral Shift-Based Method to Characterize Molecular Interactions. ASSAY and Drug Development Technologies. 2022;20:2, 83-94. https://doi.org/10.1089/adt.2021.133. PMID: 35171002