Characterization of small molecule inhibitors of the Nrf2-Keap1 interaction using MicroScale Thermophoresis

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The European Lead Factory

The European Lead Factory (ELF) is a major IMI-funded project aiming to generate new lead structures. A Joint European Compound Library (JECL) comprises >300,000 compounds from pharma companies and >150,000 synthesized bespoke for the project. European academics and SMEs can submit targets to be screened against the JECL with the potential for hit characterization and medicinal chemistry at the European Screening Centre.

Protein-protein interactions (PPIs)

PPIs can be difficult drug targets due to large, shallow interaction surfaces and are prone to high false positive hit rates from high-throughput screening (HTS). Designing a triage to validate hit target engagement helps mitigate against this and ideally includes the use of orthogonal biophysical assays. We successfully developed a label-free MicroScale Thermophoresis (MST) assay to validate inhibitors of the Nrf2-Keap1 interaction.

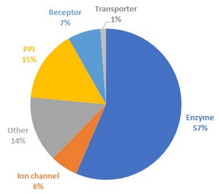
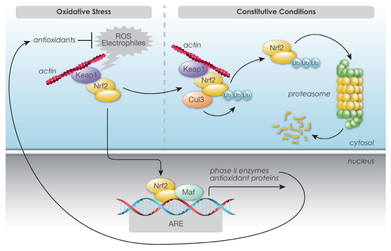


Figure 1: ELF portfolio
PPIs constitute 15% of the ELF portfolio

The Nrf2-Keap1 pathway

The complex between Keap 1 (Kelch-like ECH-associated protein) and Nrf2 (nuclear factor erythroid 2-related factor 2) regulates cyto-protective responses to oxidative stress and electrophilic agents. The Keap1-Nrf2 pathway is a therapeutic target for oxidative stress-related conditions including inflammatory, cardiovascular, neuro-degenerative diseases and cancer.

Figure 2: The Keap1-Nrf2 antioxidant pathway
Keap1 binds Nrf2 leading to ubiquitination and subsequent proteasomal degradation of Nrf2. Oxidative stress or electrophiles oxidize Keap1 reactive cysteines, leading to a conformational change, whereby Nrf2 remains bound to Keap1 but is not released. This blocks degradation of newly synthesized Nrf2, which translocates to the nucleus and promotes ARE regulated gene transcription.
Figure from Cayman Chemical.



Screening cascade

Primary Screen compounds (318132) → Confirmed hits (377) → Dose Response and redox cyclers deselection (20) → LC-MS, purity, identity (20) → Hit Selection (8)

Keap1 was screened against the JECL in a fluorescence polarisation assay (full length Keap1 and fluorescent Nrf2 peptide) with a 0.4% hit rate. Compound interfering with fluorescence or with redox activity were deselected. Eight hits remained to be characterized with label-free MST.

MicroScale Thermophoresis

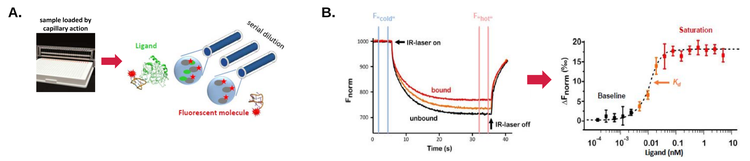
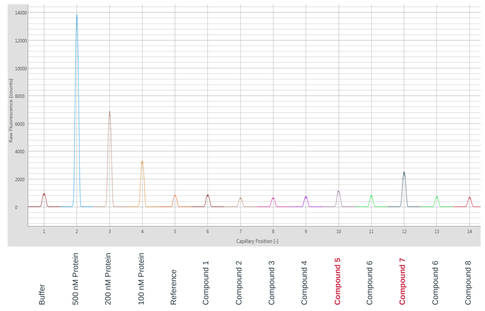


Figure 3: MST principle
A. MST uses an infrared laser to create a temperature gradient in a capillary containing a test sample. B. The movement of molecules through this temperature gradient (thermophoresis) differs when they are bound or unbound in complex, enabling estimation of the affinity of molecular interactions. The movement is measured by fluorescent dye-labelling or intrinsic fluorescence of the target protein or ligand.
Images modified from NanoTemper publications.

MST label-free pre-test

Figure 4: Target protein and test compounds were scanned using label-free MST (excitation 280nm/ emission 340 nm) to measure their intrinsic fluorescence and identify the optimal protein concentration to screen with. Compounds 5 and 7 were auto-fluorescent.



Reference and hit compound binding responses

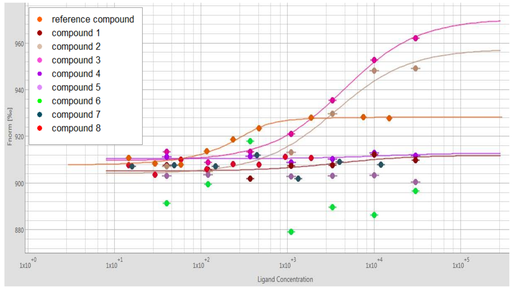


Figure 5: Testing HTS hits with MST
A reference compound and the 8 hit compounds were tested as 12-point 1 in 2 titrations with 150 nM unlabeled Keap1 protein in 50 mM Tris-HCl pH 7.4, 300 mM NaCl with 0.05% Tween-20. MST settings: 10% LED excitation power, 40% MST power and 5 second MST on-time. The reference compound and the structurally-related compounds 2 and 3 produced sigmoidal responses indicative of target binding. The other compounds appeared to be non-binders except compound 6 which produced a response characteristic of an aggregator.

Compound 3 binding is reproducible, requiring structurally-competent Keap1 protein

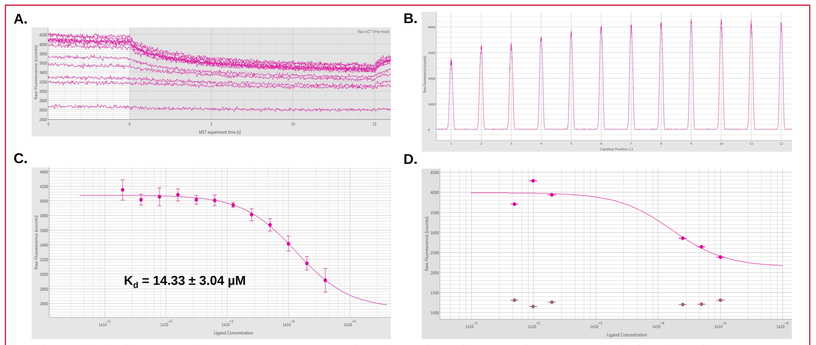


Figure 6: A. Compound 3 causes a concentration-dependent decrease in target protein baseline fluorescence, B. also observed in the capillary scan - 2-fold titration from 40 µM to 19.5 nM with 150 nM unlabeled Keap1 protein; left to right. C. Binding of compound 3 (mean +/- SD, N = 4). D. Binding was assessed against native and chemically denatured protein (pink and brown respectively).

Compound 3 competes with small molecule and peptide reference binding

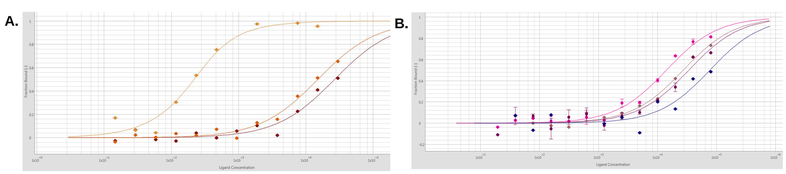
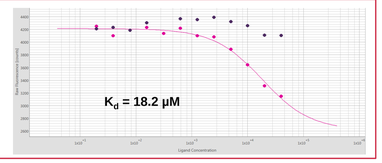


Figure 7: A. Compound 3 competes with (right-shifts) reference compound binding (0, 25 and 100 µM compound 3 from left to right). B. Nrf2 peptide mimic competes with compound 3 binding (0, 3, 10 and 30 µM peptide from left to right).

Chemical validation of compound 3 binding

Figure 8 : Compounds 2 and 3 are from the same chemical series both containing a chiral center. The opposite enantiomers showed no target binding in MST providing chemical validation of binding specificity to Keap1 and further evidence that the MST response is not due to fluorescence artefacts. Compound 3 (pink) and opposite enantiomer (dark blue).



Summary

- **Keap1 target engagement was validated by MST for two structurally related hits, compounds 2 and 3, with binding being specific to the Nrf2-Keap1 interaction interface.**
- **The MST data provided the confidence to initiate a full analogue programme resulting in >100 new compounds, leading to ligand-bound crystal structures which helped rationalize the SAR.**

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