HTRF-based kinase assay for fragment screening and MOA studies

Introduction

Research led by Dr Jeff Molkentin and Prof Michael Schneider from Imperial College has identified Nemo-Like Kinase (NLK) as a potential cardio-protective drug target. This could lead to an acute therapy to prevent cardiac muscle cell-death following myocardial infarction.

Typically, kinase inhibitors are characterised according to their binding modes, for example: as type I inhibitors that bind to the active state; or type II inhibitors that target the catalytic site in an inactive state, such as the so-called 'DFG-out' conformation.

NLK is a serine/threonine protein kinase. It is a highly divergent, atypical member of the Mitogen-activated protein kinase (MAPK) group, lacking some of the features characteristic of most mitogen-activated protein kinases. Here we describe the development of a biochemical assay suitable for fragment screening and mechanism of action studies.

Development of a Kinase screening assay

In order to ensure we were observing the correct pharmacology, we explored the kinase activity of NLK. Using the commercially-available kinase HTRF kit from Cisbio (KinEASE) we investigated the ability of in-house produced NLK to phosphorylate generic STK peptides by comparison with commercial sources of NLK from Thermofisher and Origene.

HTRF KinEASE kits use a universal biotinylated substrate, a monoclonal phosphor-specific antibody labelled with Eu3+-Cryptate, SA-XL665 and assay buffers. The KinEASE platform consists of a choice of three different kits with different substrates for Ser/Thr kinase assays (KinEASE STK S1, S2 and S3). The amount of phosphorylation of the peptide is proportional to the HTRF signal. This assay system is very robust, and tolerant of high DMSO concentrations, therefore making it suitable for fragment screening.

Our aim was to set up the assay with both high (100X the ATP K_m) and low (1 X the ATP K_m) concentrations of ATP. Initially we also compared the activity of in-house NLK with commercial sources using 3 generic peptides (figure 1)

KinEASE conditions

- Buffer: Kit buffer suppl. with 10 mM $MgCl_2$, 0.016% BSA, 0.0005% Tween, 1 mM DTT
- 1 uM Substrate 1/2/3
- 300 uM ATP
- 10-point enzyme titration (2-fold dilution, 50 nM top)

- RT for 3.5 hours

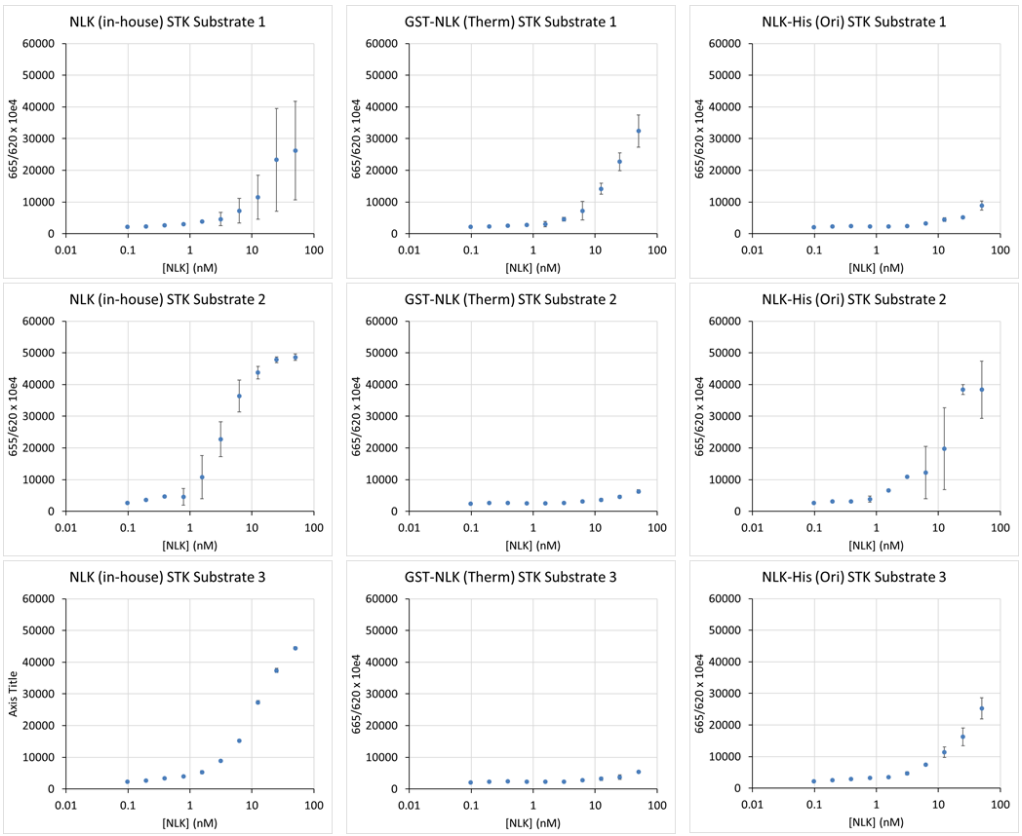


Figure 1: NLK activity assessed with KinEASE assay kit – 300 uM ATP, 3.5h incubation

In-house NLK outperformed both of the commercial sources of NLK and showed a preference for the S2 peptide (Figure 1). Therefore, S2 peptide was taken forward for further assessment by measuring ATP K_m apparent, which was determined to be ~3µM (Figure 2).

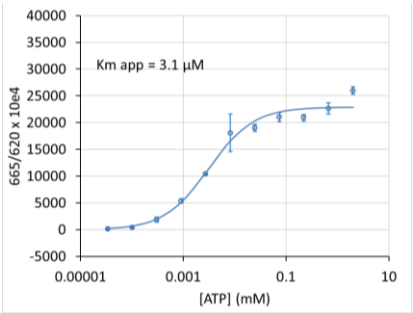


Figure 2: NLK activity assessed with KinEASE assay kit – ATP K_m Apparent. Buffer: Kit buffer suppl. with 10 mM $MgCl_2$, 0.016% BSA, 0.0005% Tween, 1 mM DTT, 1 uM Substrate 2, 6.25 nM NLK (in-house), 11-point ATP titration (3-fold dilution, 2 mM top), RT for 2 hours

Fragment screening of NLK using a KinEASE screening assay

The Domainex fragment library of *ca.* 1000 molecules was screened as singletons using the HTRF activity assay at [ATP] = 1 X and 100 X apparent K_m. Hits were defined as greater than 50% inhibition in one or more of the assays, with a total of 24 fragments identified. The fragments could be categorised based on their response to different ATP concentrations (Figure 3): Red = ATP-dependent but not competitive (Uncompetitive); Yellow = ATP-independent and not competitive (Non-competitive); and Green = ATP-dependent and competitive (Competitive inhibitors). The hits were taken forward for concentration-response analysis, and 12 fragments gave IC_{50} values.

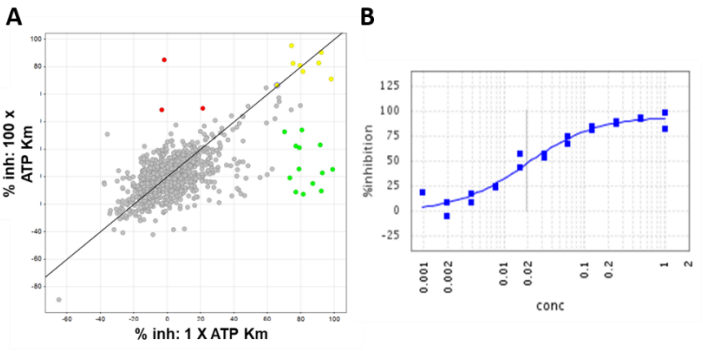
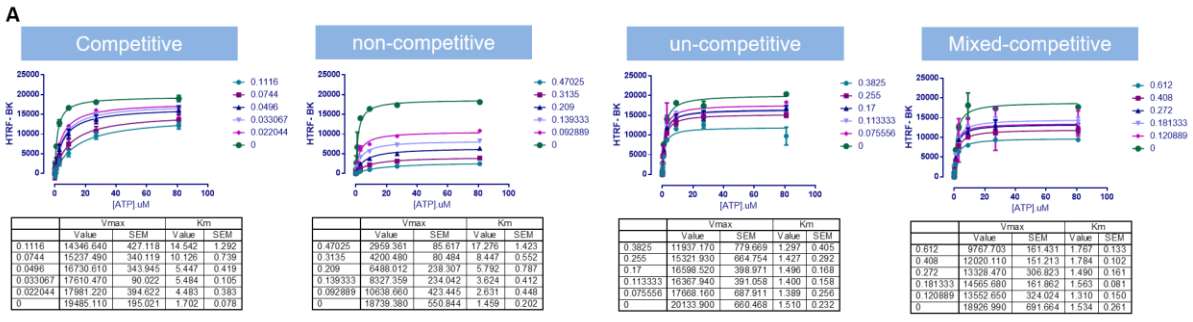


Figure 3: Fragment Screen (A) Scatter-gram for percentage inhibition of in-house NLK activity by fragments (1 mM). (B) Example dose response for key compound tested at 100 X ATP K_m showing IC_{50} 20 nM.

The mechanism of NLK inhibition for each compound can be evaluated using Michaelis-Menten analysis of ATP, K_m and V_{max} under sub-maximal concentrations of inhibitor (Figure 4). We identified four ATP competitive fragments, one un-competitive, five non-competitive and two mixed mode inhibitors. The remainder were undefined due to incomplete dose response curves.



B

Mechanism	Number hits	Best IC50
ATP Competition	4	20nM
ATP un-competitive	1	200nM
ATP Non-competitive	5	35nM
ATP mixed competitive	2	300nM
Unclear	12	

Figure 4: Michaelis-Menten analysis of a selection of inhibitors against NLK. (A) ATP is titrated against inhibitor concertation. (B) Table summarises the compound mechanism of action.

ATP competition

- Occupies the binding pocket of the enzyme in *direct competition with the substrate*.
- The enzyme can either form an [ES] complex and proceed to form the product, or it can form an [EI] complex and do nothing.
- The enzyme is not active until the inhibitor dissociates from the enzyme.
- This type of inhibition does not affect the rate of the enzyme (V_{max} unaffected), as any substrate that is bound is turned over at its normal rate, but it will hamper binding for the desired substrate (K_m increases).

ATP un-competitive

- The inhibitor binds to *the enzyme-substrate complex* (at a different [allosteric] site to the substrate).
- It forms an [ESI] complex and prevents the enzyme from processing the substrate into the product until the inhibitor dissociates. This lowers the V_{max}.
- Because the inhibitor has a preference for the [ES] complex, this reduces the overall concentration of [ES], which drives the substrate to bind to more of the free enzyme due to Le Chatelier's principle, resulting in an apparent increase in binding affinity (K_m reduced)

ATP non-competitive

- The inhibitor binds to the *free enzyme or the enzyme-substrate complex* with the same affinity (at a different [allosteric] site to the substrate), and reduces the efficiency of substrate turnover.
- This type of inhibition affects the rate of the enzyme activity (V_{max} decreases).

Mixed inhibitor

- Exhibits *preferential binding to the enzyme, or to the enzyme-substrate complex (but will bind to both), at an allosteric site*: the outcome of this is a reduced reaction rate (V_{max} reduced).
- The binding affinity of the enzyme to the substrate may be increased (decrease in K_m^{app}) if the inhibitor favours binding to the enzyme-substrate complex, or *vice versa*.