Biochemical kinase assay to improve potency and selectivity

Introduction

Research led by Prof Michael Schneider from Imperial College has identified MAP4K4 as a key player in human cardiac muscle cell death following myocardial infarction. The aim of the project was to develop a highly selective inhibitor of MAP4K4 to protect cardiomyocytes following a heart attack (for further details of this drug discovery project see our case study, MAP4K4: Kinase inhibitors for cardio-protection following heart attacks). Kinases often share a highly conserved ATP-binding pocket making it difficult to achieve selectivity, however MAP4K4 has a rare "closed" conformation of the P-Loop motif which could be exploited to gain selectivity. Here, we describe the development of a biochemical assay panel to measure the on and off target potency of MAP4K4.

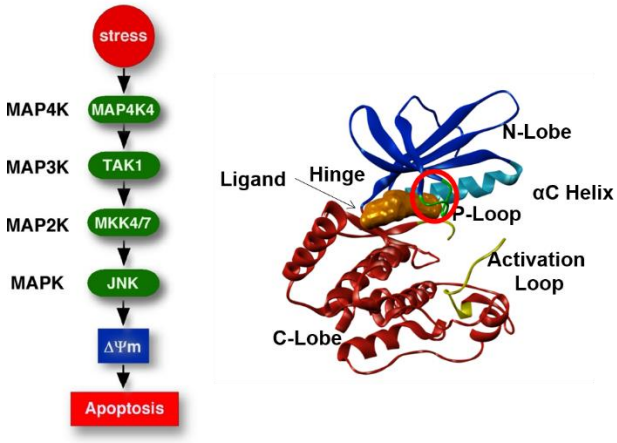


Figure 1: MAP4K4 signalling cascade triggered as a response to external stress eventually leads to cell death (left). Crystal structure representation of MAP4K4 catalytic domain in ribbon representation and ATP in surface representation. The closed P-loop conformation highlighted in red circle (right).

Assay Development

Promega ADP-Glo™ Kinase assays measure the levels of ADP generated during the course of the assay. Any residual ATP at the endpoint is degraded - using a proprietary reagent - to a non-ADP product, then the ADP is converted to ATP, which is used to produce a luminescence signal. The strength of the luminescence signal is directly proportional to the levels of ADP arising from the kinase reaction. All assays were performed at the K_m values for ATP and for the maltose binding protein (MBP) used as substrate. Substrate K_m and optimal enzyme concentration were determined prior to performing screening assays (Figure 2).

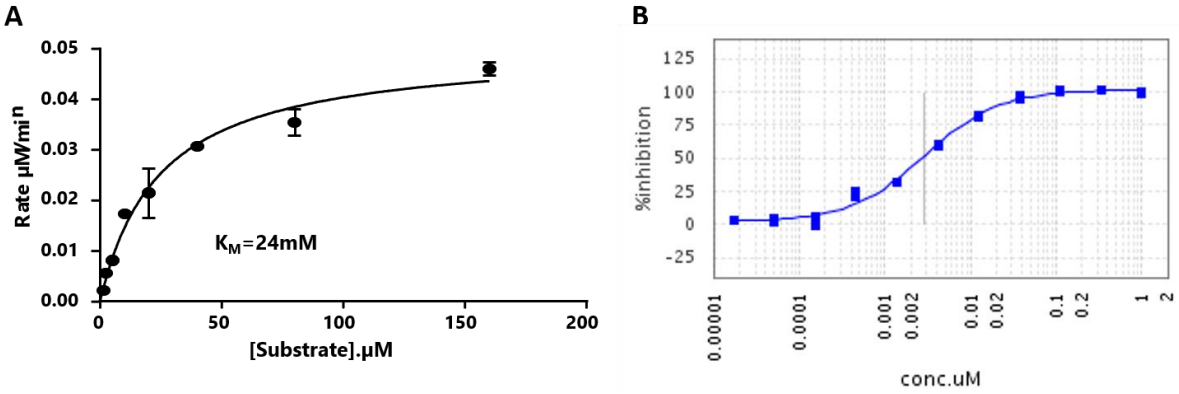


Figure 2: Assay development: (A) Example substrate titration against a fixed concentration of MAP4K4 enzyme. Turnover rate of ATP plotted against substrate concentration. (B) Example IC_{50} curve of the kinase inhibitor Staurosporine. Serial dilution of Staurosporine was incubated with a fixed concentration of enzyme, ATP and substrate at K_m. Percentage inhibition plotted against inhibitor concentration.

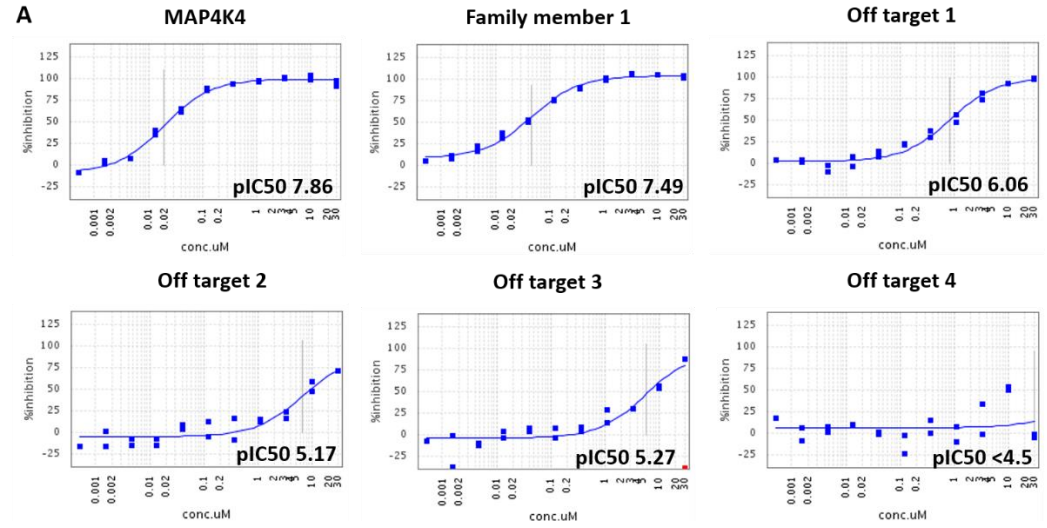
Methods

For routine screening 10-point concentration-response curves were measured in assay buffer containing 1% DMSO, and 'no-enzyme' controls were used for normalisation of inter-plate variability. Compounds and enzyme were pre-incubated for 15 min at room temperature (RT) and reaction initiated by addition of ATP and target-specific substrate at K_m. The reaction was allowed to proceed for 2 hours, before ADP was quantified using the ADP-Glo™ Kinase assay kit. To stop the reaction and remove unused ATP, the ADP-Glo™ reagent was added and incubated for 30 min at RT. Finally, the Kinase Detection Reagent was added and incubated for 30 min prior to measuring the resulting luminescence signal on a PheraStar instrument.

For an assay to pass, Z' of each plate had to be >0.7 and included controls had to be within 3-fold of historic values.

Routine Screening and Selectivity Assessment

Fifteen or more compounds were tested per week during the hit to lead and lead optimisation stages of the project. Screening of compounds against MAP4K4 and a panel of 15 off-target kinases was performed in 384-well format. The most promising compounds were profiled more widely against additional kinases, and other enzymes and receptors. An example of kinase screening data for one compound is shown in Figure 3A, and the improvement in the selectivity profile through multiple rounds of compound optimisation from 'DMXA' to 'DMXG' is shown in Figure 3B.



B

Kinase	Screening Hit	DMXA	DMXB	DMXC	DMXD	DMXE	DMXF	DMXG
MAP4K4								
Family Member 1								
Family Member 2								
Off Target 1								
Off Target 2								
Off Target 3								
Off Target 4								
Off Target 5								
Off Target 6								
Off Target 7								
Off Target 8								
Off Target 9								
Off Target 10								
Off Target 11								
Off Target 12								
Off Target 13								

pIC50 > 8
pIC50 > 7
> 100-fold selectivity
< 100-fold selectivity
< 30-fold selectivity

Figure 3: ***Routine Screening*** *(A) Inhibition curves of one MAP4K4 inhibitor during the development process for MAP4K4 and several selectivity kinases. (B) Selectivity profile improvement for selected off-target kinases across several design-make-test cycles.*