Prevention of MKK6-Dependent Activation by Binding to p38α MAP Kinase: Supporting Information

Jane E. Sullivan, Geoffrey A. Holdgate, Douglas Campbell, David Timms, Stefan Gerhardt, Jason Breed, Alexander L. Breeze, Alun Bermingham, Richard A. Pauptit, Richard A. Norman, Kevin J. Embrey, Jon Read, Wendy S. VanScyoc and Walter H. J. Ward*

AstraZeneca, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK

* Author for correspondence. E-mail: walter.ward@astrazeneca.com. Tel: ++ 44 (0)1625 515998

Expression, labelling and purification of human recombinant p38α.

DNA constructs encoding p38α (residue 2 to C-terminus), with either an N-terminal-6His-16j tag (MGSSHHHHHHLVPRGSH), or an N-terminal-cmyc-6His tag (MGSEQKLISEEDLNAHHHHHH), were cloned into a pT73.3 vector (*1*), using the *Xho*1 and *Ase*1 restriction enzyme sites. The cmyc-6His construct was used for NMR and in assays for enzyme activity and inhibitor binding. The 6His-16j construct was used for crystallography. Proteins were expressed in *Escherichia coli* BL21(DE3) strain MSD3716 in L-broth plus 10 μg/ml tetracyclin at 30 °C until an A_{550} of 0.5 was reached. Expression was induced by 0.4 mM isopropyl β-D-thiogalactopyranoside and the bacteria harvested 5 h later. Cell pastes were frozen and stored at -80 °C.

Cells were lysed by sonication (cmyc-6His-p38α) or emulsiflex (6His-16j-p38α) in 20 mM Tris pH7.5, 0.5 M NaCl, 5 mM imidazole, 0.1 mM

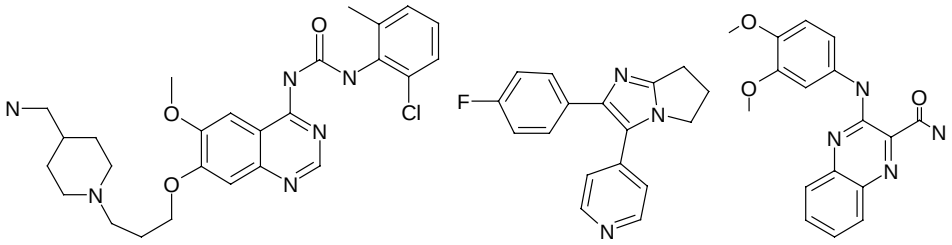
phenylmethanesulfonyfluoride, 1 μg/ml leupeptin,1 μg/ml pepstatin and 1 mg/ml lysozyme. The lysate was clarified by centrifugation and loaded onto a nickel-nitroloacetic acid superflow column. Protein was eluted in the above buffer containing 200 mM imidazole. For crystallization, the 6His-16j-p38α protein was dialysed overnight into 20 mM Tris pH7.5, 50 mM NaCl, 5% glycerol, 2 mM dithiothreitol. The 6His tag was cleaved off with thrombin (2.5 U/mg) for 7 hours at 4 °C and then stopped by addition of 0.1 mM phenylmethanesulfonyfluoride. This material was further purified on a Resource Q column (Amersham Biosciences, UK) in the same dialysis buffer, eluting with a gradient from 50 mM to 0.6 M NaCl. Further purification used a Superdex75 SEC column in 20 mM Tris pH7.5, 200 mM NaCl, 5 mM dithiothreitol. Fractions containing p38 were pooled and then concentrated in a stirred ultrafiltration cell.

Protein was labelled for NMR by growing the recombinant *E. coli* in 100% D_2O-based M9 minimal medium containing ^{15}N-ammonium chloride as the sole nitrogen source (*2*). For triply-labelled samples, U-^{13}C,^{2}H-glucose was additionally used as the sole carbon source.

Compounds used as molecular tools. As described in Experimental Procedures, specific compounds were used when determining K_d values during inhibition in solution assays (ISAs), measuring association and dissociation rate constants and inhibiting MKK6 during prevention of activation assays (Figure SI-1).

FIGURE SI-1: Structures of compounds used as molecular tools. (A) Ureidoquinazoline (*3*) target definition compound for ISA to measure binding affinity. (B) Pyridinylimidazole (*4*) fluorescent reporter for measurement of association and dissociation rate constants. (C) Quinoxaline MKK6 inhibitor (gift from Dr A. Baxter, AstraZeneca Charnwood).

(a) (b) (c)

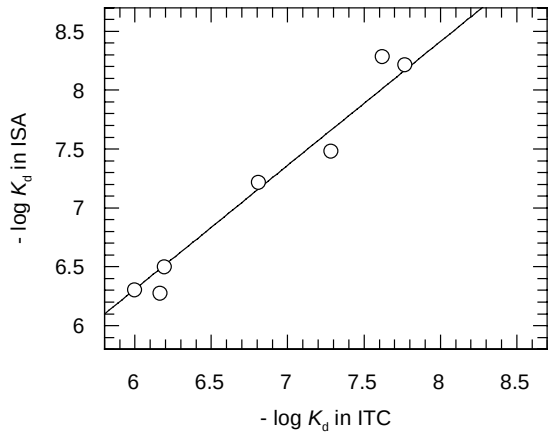


Inhibition in solution assays to measure binding affinity. A ureidoquinazoline target definition compound (Figure SI-1) was immobilised on the surface of the sensor chip. This compound competed with the binding of other inhibitors to p38α. When the enzyme was preincubated with test compounds, a proportion of it became involved in intermolecular complexes. The samples then were flowed over the sensor chip. Some of the unbound p38α associated with the immobilised target definition compound, giving a signal, which was related to the free concentration. The K_d value was estimated by fitting a dose-response relationship, described by eq (1) or (3) as shown in Figure 5 (see the main paper).

ISA was evaluated by comparing K_d values with those from isothermal titration calorimetry (*5*) under the same conditions. For compounds with calorimetrically determined K_d values from 17 nM to 1 μM, there is close agreement between the two methods (Figure SI-2). ISA has some useful

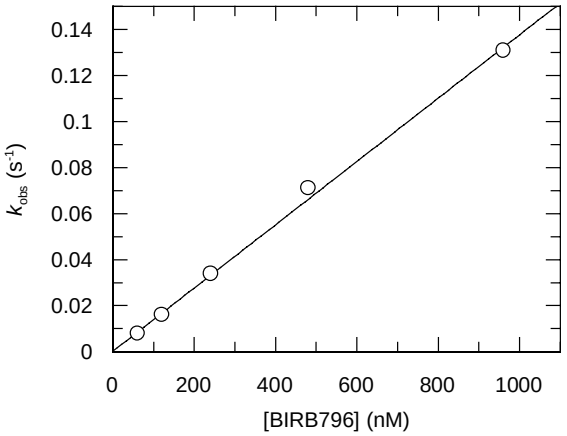
characteristics. Unlike assays of catalytic activity, it can follow binding to nonactivated enzyme. ISA consumes over 100-fold less protein than isothermal titration calorimetry.

FIGURE SI-2: Comparison of binding affinities determined using ISA and isothermal titration calorimetry. The best fit line (slope = 1.05, 95% CIs 1.02-1.08) assumes a linear relationship between log K_d values, with an intercept that passes through the origin.



Binding kinetics for BIRB796. The results in Figure SI-3 are discussed in the main paper.

FIGURE SI-3: Rate of binding BIRB796 to nonactivated p38α. Details are in Experimental Procedures. The best-fit line is shown using k_{on} = 1.4 x 10^5 s^{-1} M^{-1} (95% CIs within 2-fold), k_{off} = 2 x 10^{-4} s^{-1} (95% CIs 0.08 to 50 x 10^{-4} s^{-1}).



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