



## X-ray crystal structure of JNK2 complexed with the p38 $\alpha$ inhibitor BIRB796: Insights into the rational design of DFG-out binding MAP kinase inhibitors

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### ABSTRACT

JNK2 and p38 $\alpha$  are closely related mitogen-activated protein kinases that regulate various cellular activities and are considered drug targets for inflammatory diseases. We have determined the X-ray crystal structure of the clinical phase II p38 $\alpha$  inhibitor BIRB796 bound to its off-target JNK2. This shows for the first time a JNK subfamily member in the DFG-out conformation. The fully resolved activation loop reveals that BIRB796 inhibits JNK2 activation by stabilizing the loop in a position that does not allow its phosphorylation by upstream kinases. The structure suggests that substituents at the BIRB796 morpholino group and modifications of the *t*-butyl moiety should further increase the p38 $\alpha$  to JNK2 potency ratio. For the design of selective DFG-out binding JNK2 inhibitors, the binding pocket of the BIRB796 tolyl group may have the best potential.

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Mitogen-activated protein (MAP) kinases play key roles in the transmission of signals from cell surface receptors to transcription factors which upregulate the expression of pro-inflammatory cytokines. The MAP kinase p38 $\alpha$  plays a key role in the biosynthesis of TNF- $\alpha$ , IL-1, IL-6 and IL-8, and has been found to induce the production of COX-2, MMP-1 and MMP-3.<sup>1,2</sup> c-Jun N-terminal kinase (JNK), also a MAP kinase, is involved in the production of phospho-c-Jun and AP-1 as well as the upregulation of MMP-1, MMP-3 and MMP-13.<sup>3,4</sup> Redundant and non-redundant functions of the JNK isoforms JNK1 and JNK2 in the immune system and arthritis have been described.<sup>5</sup> Consequently, both p38 $\alpha$  and JNK have been targeted for the discovery of small-molecule therapeutics for inflammatory diseases.<sup>6–8</sup>

The diaryl urea p38 $\alpha$  inhibitor BIRB796 (Fig. 1A,  $K_D = 0.16$  nM) blocks TNF- $\alpha$  production in cell cultures and it is efficacious in both collagen-induced arthritis and endotoxin-stimulated TNF- $\alpha$  release animal models.<sup>9</sup> BIRB796 also has been shown to be a strong inhibitor of TNF- $\alpha$  production in lipopolysaccharide-induced endotoxemia, a model for inflammation in humans.<sup>10</sup> Published results of phase I<sup>6</sup> and phase II<sup>11</sup> clinical trials in Crohn's disease and rheumatoid arthritis have been summarized recently. BIRB796 also binds with high affinity to JNK2 ( $K_D = 4.6$  nM)<sup>12</sup> and with lower affinity to JNK3 ( $K_D = 62$  nM), but it shows no significant binding to JNK1.<sup>13</sup> In HCA2 cells, BIRB796 at least partially inhibits total activity of the JNK subfamily at concentrations higher than 1.0  $\mu$ M.<sup>14</sup> It has been reported that the pharmacological activity of BIRB796 is mainly due to its p38 $\alpha$  inhibitory activity.<sup>12</sup> To what extent the JNK2 activity of BIRB796 is responsible for the potentially drug-related adverse events observed in clinical trials, for example, infections and rashes,<sup>15</sup> is unclear.

The X-ray crystal structure of p38 $\alpha$  in complex with BIRB796 revealed that this inhibitor blocks p38 $\alpha$  signaling by stabilizing the 'DFG-out' conformation of p38 $\alpha$ ,<sup>9</sup> which makes it a so-called 'type II' kinase inhibitor. This conformation does not allow ATP binding to the enzyme, and it is typically associated with slow

*Abbreviations:* JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein.

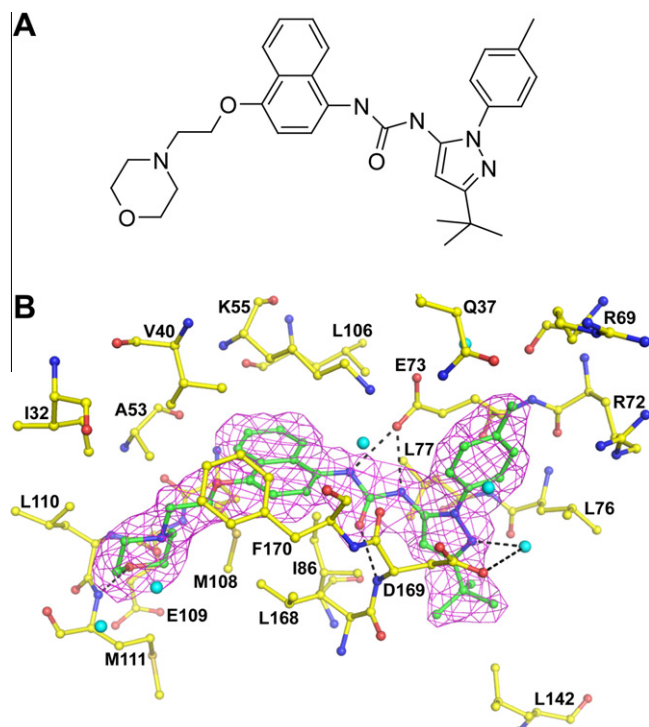
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**Figure 1.** X-ray crystal structure of JNK2 with BIRB796 bound. (A) Structural formula of BIRB796. (B) Ball-and-stick representation of JNK2 residues (yellow) within 4 Å of BIRB796 (green). Water molecules are displayed in cyan. Protein-ligand H-bonds are indicated as black hashes. A  $2F_o - F_c$  electron density map contoured at  $1\sigma$  is displayed in magenta.

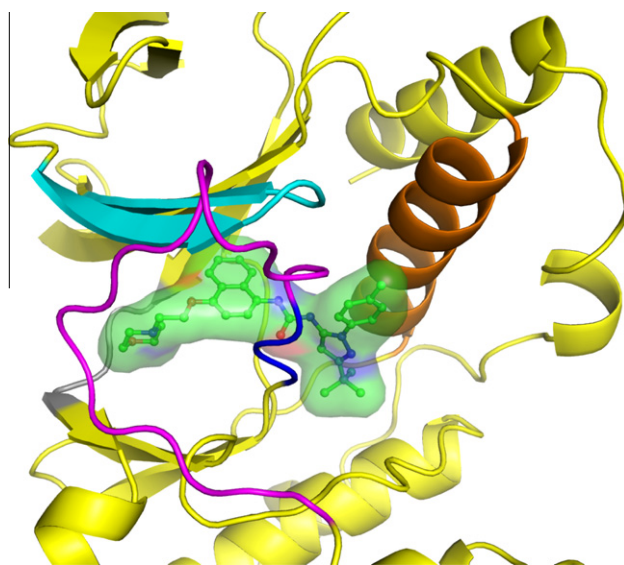
binding kinetics. It has been postulated that protein kinase inhibitors that bind to the DFG-out conformation provide a better starting point for the design of highly selective inhibitors compared to ‘type I’ inhibitors that bind to the ‘DFG-in’ conformation.<sup>16–18</sup> Consequently, different screening methods that allow identification of DFG-out binders have recently been developed and successfully deployed for p38 $\alpha$ .<sup>19,20</sup> JNK specific DFG-out inhibitors, however, have not been reported to our knowledge, and a crystal structure of JNK in the DFG-out conformation is not available in the public domain. This prompted us to determine the X-ray crystal structure of JNK2 in complex with BIRB796. This structure will facilitate the design of JNK2 selective diaryl urea inhibitors in particular and DFG-out binders in general. The structural information obtained also allows the rational design of BIRB796 analogues as p38 $\alpha$  inhibitors with decreased JNK affinity, which may improve the therapeutic potential for this compound class.

Untagged JNK2 $\alpha$  kinase domain comprising amino acids 1–364, the JNK3 chimera mutations D3K, C6V, S8N and Q14E, and the surface mutations K203A, E204A, K250A and K251A was over-expressed in *Escherichia coli* and purified following a protocol described previously.<sup>21</sup> These amino acid exchanges occur at least 12 Å away from the binding site of the BIRB796 molecule and therefore do not affect its binding to JNK2. Remarkably, the set of JNK2 surface mutations required for the co-crystallization of BIRB796 is different from that used to co-crystallize the indazole inhibitor furan-2-carboxylic acid {3-[5-(4H-[1,2,4]triazol-3-yl)-1H-indazol-3-yl]-phenyl}-amide.<sup>21</sup> The mutant JNK2 protein at a concentration of 10 mg/ml (25 mM HEPES (pH 7.5), 0.1 M sodium chloride, 0.01 M magnesium chloride, 5% glycerol, 1 mM TCEP, 0.02% Octyl- $\beta$ -glucopyranoside) was incubated with 0.1 mM BIRB796 and 5% DMSO. The protein–ligand complex was crystallized by vapor diffusion in hanging drops at 20 °C. Protein solution (0.7  $\mu$ l) was mixed with 0.7  $\mu$ l of 0.1 M HEPES (pH 7.5), 0.1 M

sodium chloride and 1.6 M ammonium sulfate. Crystals were transferred to a buffer containing 30% sucrose, 1.8 M ammonium sulfate, 0.1 M HEPES (pH 7.5), 0.1 M BIRB796 and 5% DMSO for cooling in liquid nitrogen. X-ray diffraction data were collected by Reciprocal Space Consulting, LLC (Oakland, California, USA) at the synchrotron beam line 9-2 of the Stanford Synchrotron Radiation Laboratory (Palo Alto, California, USA) using an ADSC Quantum 315R CCD detector. The diffraction images were processed using DENZO and the intensities were scaled using SCALEPACK<sup>22</sup> to 2.35 Å resolution with good statistics: completeness = 99.9% (100% in highest resolution shell),  $I/\sigma(I)$  = 11.1 (2.1),  $R_{\text{sym}}$  = 8.4% (56.9%),  $R/R_{\text{free}}$  = 21.4/25.4%. Molecular replacement was performed with the program PHASER<sup>23</sup> using the crystal structure of JNK2 complexed with the indazole inhibitor furan-2-carboxylic acid {3-[5-(4H-[1,2,4]triazol-3-yl)-1H-indazol-3-yl]-phenyl}-amide (PDB accession number 3E7O)<sup>21</sup> as search model. The solution found was a JNK2 dimer in space group  $P2_12_12_1$  ( $a$  = 76.2 Å,  $b$  = 92.3 Å,  $c$  = 112.1 Å). This new crystal form should allow the co-crystallization of JNK2 with DFG-out binding inhibitors in general. The model was refined against the experimental data and electron density maps were calculated using REFMAC5.<sup>24</sup> The electron density for BIRB796 was well defined and it could be fitted unambiguously (Fig. 1B). The structure model was built with the graphics software MOLOC<sup>25</sup> and illustrations of the final crystal structures were created using Pymol (<http://www.pymol.org/>). Coordinates and structure factor amplitudes have been deposited to the Protein Data Bank with accession number 3NPC.

The crystal structure described in this manuscript shows how BIRB796 binds to the DFG-out conformation of JNK2 (Fig. 2). The entire activation loop (amino acids 172–188) and the adjacent DFG sequence are resolved. D169, F170 and G171 swing out and open a pocket adjacent to helix C for the *t*-butyl-pyrazole moiety of BIRB796 (amino acids 64–79). The activation loop is stacking against the Gly-rich loop (amino acids 30–42) thereby shielding BIRB796 from solvent.

The secondary structure elements around the ATP binding pocket in the crystal structure of JNK2 complexed with BIRB796 adopt conformations distinctly different from those observed for JNK2 complexed with the indazole inhibitor furan-2-carboxylic acid {3-[5-(4H-[1,2,4]triazol-3-yl)-1H-indazol-3-yl]-phenyl}-amide (Fig. 3).<sup>21</sup> The Gly-rich loop in the indazole-bound structure is positioned as much as 5 Å higher and away from the bound ligands than in the



**Figure 2.** Ribbon representation of JNK2 around the BIRB796 binding site. Cyan: Gly-rich loop, orange: helix C, dark blue: DFG, magenta: activation loop. BIRB796 is shown in ball-and-stick and molecular surface representation in green.

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