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Allosteric modulation of JNK docking-site interactions with ATP-competitive inhibitors

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Abstract

The c-Jun N-terminal kinases (JNKs) play a wide variety of roles in cellular signaling processes, dictating important, and even divergent, cellular fates. These essential kinases possess docking surfaces distal to their active sites that interact with diverse binding partners, including upstream activators, downstream substrates, and protein scaffolds. Prior studies have suggested that the interactions of certain protein binding partners with one such JNK docking surface—termed the D-recruitment site (DRS)—can allosterically influence the conformational state of the ATP-binding pocket of JNKs. To further explore the allosteric relationship between the ATP-binding pockets and DRSs of JNKs, we investigated how the interactions of the scaffolding protein JIP1, as well as the upstream activators MKK4 and MKK7, are allosterically influenced by the ATP-binding site occupancy of the JNKs. We show that the affinity of the JNKs for JIP1 can be divergently modulated with ATP-competitive inhibitors, with a greater than 50-fold difference in dissociation constant observed between the lowest and highest affinity JNK1-inhibitor complexes. Furthermore, we found that we could promote or attenuate phosphorylation of JNK1's activation loop by MKK4 and MKK7, by varying ATP-binding site occupancy. Given that JIP1, MKK4, and MKK7 all interact with JNK DRSs, these results demonstrate that there is functional allostery between the ATP-binding sites and DRSs of these kinases. Furthermore, our studies suggest that ATP-competitive inhibitors can allosterically influence the intracellular binding partners of the JNKs.

Graphical Abstract

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“Modulation of JNK docking-site interactions with ATP-competitive inhibitors” *Chloe K. Lombard, Audrey L. Davis, Takayuki Inukai, and Dustin J. Maly**

Introduction

The c-Jun N-terminal kinases (JNKs) are a multi-functional subfamily of the mitogen activated protein kinases (MAPKs) that play critical roles in cell migration, differentiation, proliferation, and apoptosis.¹⁻⁵ The JNK family consists of three distinct but highly homologous isoforms, JNK1, JNK2, and JNK3, which have more than ten total splice variants.^{5,7} JNK1 and JNK3 share 92% sequence similarity and only possess one residue difference in the 23 residues comprising their ATP-binding sites. JNK1 and JNK2 share 83% sequence similarity and also only possess one residue difference in the 23 residues comprising their ATP-binding sites.⁸⁻¹⁰ JNK1 and JNK2 are expressed ubiquitously, while JNK3 is expressed mainly in the brain, heart, and testes. JNKs exist in a predominately inactivate, non-phosphorylated state, but are activated by a variety of extracellular stimuli, including UV radiation, oxidative stress, and inflammatory cytokines.^{5, 6, 11} Following cellular stimulation, JNKs are predominately activated by their upstream MAPK kinases (MKKs), MKK4 and/or MKK7, via dual phosphorylation of the Thr/Pro/Tyr (TPY) motifs within their activation loops.¹¹⁻¹³ Activated JNKs then phosphorylate a variety of downstream substrates.^{5, 6, 11} Due to their contribution to diverse signaling pathways, which can often determine cell fate, JNK activity is tightly controlled. Aberrant JNK signaling has been associated with cancer, insulin resistance, neurodegeneration, and the development of autoimmune conditions.^{5, 14-21}

The structural architecture of JNKs is relatively simple, consisting of a single bi-lobal kinase domain with an ATP-binding cleft at its center. Phospho-acceptor substrates of JNKs bind in an extended conformation, adjacent to the JNK ATP-binding site. While JNKs display substrate preferences based on the primary sequence of the phospho-acceptor, multiple docking surfaces-distal to the site of phosphate transfer-are utilized to discriminate between cellular substrates.^{5, 11, 22, 23} These docking interactions provide enhanced specificity in signaling. Many JNK substrates, and other protein binding partners, contain short interacting motifs known as docking-domains (D-domains). D-domains typically consist of a few basic residues and a hydrophobic (ϕ) motif, surrounding a one to six residue spacer ($K/R_{2-3}-X_{1-6}-\phi-X-\phi$).^{24, 25} Proteins with D-domains bind to JNK D-recruitment sites (DRS), which are located on the face opposite of the ATP-binding site, mainly on the C-terminal lobe (Figure

1A). The DRSs of JNKs are multi-functional and mediate interactions with scaffolds, upstream activating MKKs, and a number of substrates.^{22-24, 26, 27}

There is believed to be allosteric communication between the ATP-binding sites and DRSs of JNKs, although the full extent of this communication has not yet been determined. Evidence to suggest the existence of allostery between the DRSs and ATP-binding sites of JNKs lies largely in studies exploring the interactions of JNK interacting proteins (JIPs). JIPs are a family of scaffold proteins that have been shown to interact directly with the DRSs of JNKs.^{28, 29, 30} Prior work identified an eleven amino acid peptide, derived from the D-domain of JIP1 (RPKRPTTLNLF; JIPtide), that is capable of interacting with the DRSs of JNKs, like the full-length scaffold.³¹ Structural alignments of *apo*-JNK3 with either JNK1-JIPtide or JNK3-JIPtide complexes, shows that the N-terminal lobes of JIPtide-bound JNKs are rotated ~15° relative to the C-lobe (Figure S1).^{10, 32} Additionally, comparisons of *apo*-JNK3 and JNK3 complexed with other D-domain peptides—derived from JNK substrates—also show significant conformational differences.³² Furthermore, JIPtide binding has been demonstrated to modestly affect the affinities of JNKs for ATP.^{10, 33, 34} Given that the conformation of kinase ATP-binding sites can be modulated by ligand binding, we were curious whether ATP-competitive inhibitors could alter the ability of both scaffold proteins and upstream MKKs to interact with and act upon the JNKs, by allosterically modulating the behavior of their DRSs. To probe this, we selected ten diverse ATP-competitive inhibitors of the JNKs and systemically probed how they influence the behavior of JIP1, MKK4, and MKK7 (Figure 1B). We found that ATP-competitive compounds can either *enhance* or *diminish* JNK-JIP1 interactions, and either *activate* or *attenuate* activation by MKK4 or MKK7.

Materials and Methods

Cloning and mutagenesis.

Bacterial expression plasmids containing genes encoding His₆-JNK1α1 (JNK1), His₆-SUMO-JNK1α1 (His₆-SUMO-JNK1), His₆-JNK2α1 (JNK2), His₆-JNK3α1 (JNK3), His₆-SUMO-MKKβ (MKK4), His₆-SUMO-MKK7β1 (MKK7), Flag-JIP1b-His₆ (JIP1), into pMCSG7 vectors were created using Gibson assembly.³⁵ The genes for MKK4β, MKK7β1, and Flag-JIP1b were obtained as gifts from Roger Davis and were provided in pcDNA3 (Addgene plasmid #s MKK4β: 15517; MKK7β1: 14622; Flag-JIP1b: 52123). The gene for His₆-Ulp1 (Ulp1) was synthesized as a G-block (Integrated DNA Technologies). The pTH1-SaSrtA-A (Sortase A) plasmid was obtained as a gift from Teruyuki Nagamune (Addgene plasmid # 64979). His₆-p38α and His₆-GST-MAP3K1 were provided by Hari, *et al.* in bacterial expression vectors.^{36, 37} Uniprot IDs, DNA, and protein sequences for constructs we modified are provided in Table S1.

Protein expression and purification (Figure S2).

His₆-JNK1α1, His₆-SUMO-JNK1α1, His₆-JNK2α1, His₆-JNK3α1, His₆-SUMO-MKK4β, His₆-SUMO-MKK7β1, His₆-MAP3K1, His₆-p38α, Flag-JIP1b-His₆, His₆-Ulp1, and His₆-Sortase A were expressed in *Escherichia coli* BL21(DE3) cells in LB Miller broth. Cells were induced between OD₆₀₀ 0.65-0.75 with 400 μM isopropyl β-D-thiogalactoside at 18 °C

overnight. All purification steps were carried out at 4 °C. Cells were lysed with sonication in 2 mL/gram pellet weight of wash/lysis buffer consisting of 50 mM HEPES (pH 7.5), 300 mM NaCl, 20 mM imidazole, 10% glycerol and 1 mM phenylmethylsulfonyl fluoride. The lysate was centrifuged at 10000 g for 20 min and the supernatant was allowed to batch bind for 60 min with 0.4 mL of cell culture of Ni-NTA (Ni²⁺-nitrilotriacetate) resin. The resin was collected by centrifugation at 500 g for 5 min and washed with 20 mL of wash/lysis buffer per liter of culture. The wash step was repeated three times. The protein was eluted using ~4 mL of elution buffer (50 mM HEPES (pH 7.5), 300 mM NaCl, 200 mM imidazole and 10% glycerol) per liter of culture. Then, the eluate was dialyzed against 50 mM HEPES (pH 7.5), 200 mM NaCl, 5% glycerol and 1 mM dithiothreitol (DTT). His₆-JNK1α1, His₆-SUMO-JNK1α1, His₆-JNK2α1, and His₆-JNK3α1 were expressed in their inactive forms. The aliquoted proteins were flash-frozen and stored at -80 °C. His₆-SUMO-MKK7β1 was co-expressed with MAP3K1, to allow purification of the active MKK7β1 which was then subjected to overnight Ulp1 cleavage. His₆-SUMO-MKK4β was expressed alone and subsequently activated by MAP3K1 in vitro, during overnight Ulp1 cleavage. Flag-JIP1b-His₆ was purified using sequential Ni-NTA and Anti-Flag purification. After elution from Ni-NTA, Flag-JIP1b-His₆ was bound to Anti-Flag M2 magnetic beads (Sigma), washed twice using 5 CV of TBS (50 mM tris(hydroxymethyl)aminomethane (Tris)/HCl (pH 7.5), 150 mM NaCl), and eluted using 1 mg/mL 3X Flag peptide (MDYKDHDGDYKDHDIDYKDDDDK) in TBS. His₆-SUMO-JNK1α1 was treated with Ulp1 during O.N. dialysis to yield Gly-JNK1α1 for subsequent Sortase A labeling.

Sortase A labeling of Gly-JNK1α1 (Figure S3).

Labeling was completed using a method modified from Theile, et al. (2013).⁴⁷ After O.N. dialysis, Gly-JNK1α1 was dialyzed against 1 mM β-mercaptoethanol (BME), and the His₆-Ulp1 (used to generate the free terminal Gly-JNK1α1) and cleaved His₆-SUMO, were removed by Ni-NTA. 10 μM of Gly-JNK1α1 was combined with 20 μM Sortase A and 200 μM TMR-LPETGG (TMR: 6-carboxytetramethylrhodamine) in dimethyl sulfoxide (DMSO; 4% v/v final in assay) in Sortase A labeling buffer (50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 20 mM imidazole, 10% glycerol, and 1 mM BME). The labeling took place over 1.5 hr at 4°C in the dark. The His₆-Sortase A was then removed by Ni-NTA and the remaining peptide was removed using a Zeba column (Thermo Fisher), which also allowed exchange into our standard dialysis buffer, containing 1 mM DTT.

Peptides.

All peptides were synthesized by Genscript and purified by High Performance Liquid Chromatography (HPLC). The peptide for the fluorescence polarization assays, containing the D-domain of JIP1 (JIPTide), was synthesized with a TMR label at the N-terminus and a free acid at the C-terminus (TMR-JIPTide). The sequence for the JIP 11-mer was RPKRPTTLNLF (amino acids 153–163 of JIP1). The Sortase A peptide for the N-terminal labeling of JNK1α1 was synthesized with a TMR label at the N-terminus and a free acid at the C-terminus (TMR-LPETGG).

Instrumentation.

FP and TR-FRET assays were conducted using 384-well plates and detected using a PerkinElmer Envision 2104 Multi-label Reader. Western blots were imaged on a LiCor Odyssey gel image scanner using both 680 nm and 780 nm channels.

Binding Assays.

To measure binding of the compounds to the ATP-site of non-phosphorylated His₆-JNKs, serial dilutions (1:3) of compounds in DMSO (4% v/v final in assay) were prepared at 750 μ M starting (30 μ M final in assay). Compounds were added to 33 nM JNKs in buffer (50 mM Tris/HCl (pH 8), 100 mM NaCl, 100 nM SCP2-Cy5 (Figure 2), and 1 nM europium Anti-His₆ in a 384-well plate (20 μ L per well). TR-FRET was determined using an excitation wavelength of 320 nm and emission wavelengths of 615 nm (Eu³⁺) and 665 nm (Cy5). Compounds were titrated in triplicate and JNK-compound K_{DS} were determined, and subsequently the K_S , using the K_D of the probe for JNK1 α 1 (0.39 $\pm$ 0.24 μ M).

Activation of JNK1 α 1 for activity assays.

JNKs were activated using phospho-MKK4 α . 900 nM JNK1 α 1 or 2.7 μ M JNK2 α 1 were pre-activated with 150 nM MKK4 α for 1 hr at RT in (50 mM Tris/HCl (pH 7.5), 0.01% (v/v) Tween 20, 10 mM MgCl₂, 2 mM DTT, 1 mM Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.1 mg/mL BSA) with 400 μ M ATP.

TR-FRET based activity assays.

The activated JNKs were tested using the Lance Ultra TR-FRET assay (Perkin Elmer). Serial dilutions (1:3) of compounds in DMSO (4% v/v final in assay) were prepared at a 750 μ M starting concentration (diluted to 30 μ M final in the assay). Compounds were added to 15 nM activated JNK1 α 1 or 25 nM JNK2 α 1 in buffer (50 mM Tris/HCl (pH 7.5), 0.01% (v/v) Tween 20, 10 mM MgCl₂, 2 mM DTT, 1 mM EGTA, 0.1 mg/mL BSA), with ATP. For the JNK2 α 1 assays, 100 μ M ATP was used for all compounds. For the JNK1 α 1 assays, 100 μ M ATP was used for compounds **1-9** and 1 mM ATP for compound **10**. JNKs were pre-incubated for 30 min with ATP-competitive inhibitors and ATP. To initiate the reaction, ulight-labeled myelin basic peptide (ulight-MBPtide) was added (300 nM for JNK1 α 1 and 150 nM for JNK2 α 1). The reaction mixture was incubated in a volume of 15 μ L per well in a 384-well plate at room temperature for 4 hr, then quenched with 10 mM ethylenediaminetetraacetic acid (EDTA) in modified Lance Detect Buffer (40 mM Tris-HCl (pH 7.5) and 100 mM NaCl). After a 5 min incubation with the quench reagents, 0.5 nM europium labeled Anti-phospho-MBPtide was added in Lance Detect Buffer. After 1 h of incubation, the plates were read on an Envision Multi-label Reader. TR-FRET was determined using an excitation wavelength of 320 nm and emission wavelengths of 615 nm (Eu³⁺) and 665 nm (ulight-MBPtide). Relative amounts of activity were calculated using the ratio of 665 nm light to 615 nm light. Titrations were run in triplicate and JNK-inhibitor IC₅₀s were determined.

FP assays.

To measure the binding of the JIP1 11-mer (JIPTide) with JNKs ($\pm$ ATP-competitive compound), 30 μ M compounds were pre-incubated with non-phosphorylated JNKs and 30 nM TMR-JIPTide in a 384-well plate (30 μ E per well) for 60 min at room temperature in the assay buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM MgCl₂, 0.005% Brij-35, 0.1% 2-mercaptoethanol and 0.05% bovine serum albumin (BSA)). A total of 6 JNK concentrations (above and below the K_D s) were screened in triplicate, and JNK-JIPTide K_D s ($\pm$ ATP-competitive compound) were determined. FP assays used 595 nm excitation and emission filters.

Full length JNK1 α 1-JIP1b pull-downs.

Each pull-down contained 20 μ L of Anti-Flag M2 magnetic beads (Sigma) in 120 μ L total volume. Full length Flag-JIP1b was bound to the beads at a final concentration of 0.6 μ M throughout the pull-down (or Flag-Grb2 as a control scaffold; see Figure S4). JNK1 α 1 was incubated with the JIP1b bound beads, in buffer (50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.01% (v/v) Tween 20, 10 mM MgCl₂, and 0.1 mg/mL BSA), containing 10 μ M ATP-competitive compound with 4% (v/v final) DMSO (or DMSO alone as a control). Either 500 nM or 200 nM JNK1 α 1 was allowed to bind JIP1b for compounds that disrupt or enhance binding, respectively, for 1 hr at RT. Free JNK1 α 1 was washed from the pull-downs using three sequential washes with TBS (50 mM Tris/HCl (pH 7.5), 150 mM NaCl). The remaining JNK1 α 1-JIP1b was eluted using 1 mg/mL 3X Flag peptide in TBS. JIP1b and JNK1 α 1 were detected by western blotting for Flag and total JNK, respectively.

Activation of JNKs by MKK4 β /MKK7 β 1.

Phosphorylation of TMR-JNK1 α 1 was carried out in 60 μ L reactions containing 200 nM JNK1 α 1 in buffer (50 mM Tris/HCl (pH 7.5), 0.01% (v/v) Tween 20, 10 mM MgCl₂, and 0.1 mg/mL BSA), 400 μ M ATP, 10 μ M ATP-competitive compound in DMSO (or DMSO alone as a control) 4% (v/v final), and either activated MKK4 β or MKK7 β 1, at concentrations in the linear range for either the dual or mono phosphorylation event. These concentrations for MKK7 β 1 were 150 nM and 0.25 nM, respectively. These concentrations for MKK4 β were 250 nM and 0.5 nM, respectively. There was a 30 min pre-incubation of JNK1 α 1, ATP-competitive compound, and ATP before starting the assay by addition of MKKs. The reactions proceeded for 1 hr. TMR-JNK1 α 1 was separated using Phos-tag SDS-PAGE and imaged using fluorescence.

Phos-tag SDS-PAGE.

Gels were freshly prepared on the day of the experiment using a Biorad mini-PROTEAN Tetra Handcast system. Resolving gels contained 0.35 M bis-Tris/HCl (pH 6.8), 7% acrylamide (37.5:1 acrylamide: bis-acrylamide), 0.1 mM MnCl₂, 0.05 mM Phos-tag acrylamide, 0.05% ammonium persulfate, and 0.001% TEMED. Stacking gels contained 0.35 M bis-Tris/HCl (pH 6.8), 4% acrylamide (37.5:1 acrylamide: bis-acrylamide), 0.05% ammonium persulfate, and 0.001% TEMED. Each layer was allowed to polymerize for 1 hr at RT. Gels were run in buffer (pH 7.8) containing 50 mM MOPS, 50 mM Tris base, 0.1% SDS, and 5 mM sodium bisulfite (added fresh from a 1 M solution) at 150 V. Samples were

prepared in EDTA-free SDS loading buffer and throughout sample generation, EDTA was avoided to prevent distortion of the protein's travel. All wells were loaded with an equal volume of sample, containing similar buffer conditions. Unused wells were filled with mock samples containing buffer and loading dye.

MKK4 β inhibition assays using p38 α as a substrate.

Kinase assays for MKK4 β were conducted in 30 μ L assay volume containing the compound in DMSO (4% v/v final in assay), 100 nM MKK4 β , 0.007 μ Ci/ μ L [γ -³²P]ATP, and 3.9 μ M p38 α as a substrate in assay buffer (50 mM Tris/HCl (pH 7.5), 0.01% (v/v) Tween 20, 10 mM MgCl₂, 2 mM DTT, 1 mM EGTA, 0.05 mg/mL BSA). Kinase activity was first determined to be linear at 100 nM MKK4 β . Assays were run for 2 hr at RT and quenched by spotting 4.6 μ L of each reaction onto phosphocellulose membranes (Reaction Biology). The membranes were subjected to three sequential washes in 0.5% phosphoric acid for 10 min, dried, and exposed O.N. to a phosphor screen (GE Healthcare). Blots were scanned using a phosphor scanner (GE Typhoon FLA 9000). Spots were quantified using ImageQuant software.

MKK4 β pull-downs.

Each pull-down contained 20 μ L of Ni-NTA in 120 μ L total volume. Full length His₆-SUMO-MKK4 β was bound to the beads at a final concentration of 0.6 μ M throughout the pull-down. JNK1 α 1 was incubated with the MKK4 β bound beads in buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM imidazole) containing 30 μ M compound with 4% (v/v final) DMSO (or DMSO alone as a control). 500 nM JNK1 α 1 was allowed to bind MKK4 β , for 1 hr at RT. Free JNK1 α 1 was washed from the pull-downs using three sequential washes with buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM imidazole). The remaining JNK1 α 1-MKK4 β was eluted using buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 200 mM imidazole). MKK4 β and JNK1 α 1 were detected by western blotting for MKK4 and total JNK, respectively.

Data Analysis.

For all experiments, there were linear relationships between signal and enzyme concentration or time. IC₅₀s and K_Ds were fitted to data by unweighted nonlinear regression using GraphPad Prism. When fitting equations to data, the parameter values, x (concentration of enzyme or compound), were replaced by log₁₀x. Data was reported as Average $\pm$ S.E.M.

Results and Discussion

A diverse panel of ATP-competitive inhibitors that bind to activated and non-activated JNKs.

To facilitate our investigation of how ATP-binding site occupancy affects the docking interactions of JNKs, we selected a panel of ten inhibitors based on their structural diversity, predicted ability to form varied contacts with different regions of JNK ATP-binding sites, and the capacity to interact with all three JNK isoforms (Figure 1B). Because we were interested in exploring how JIP1 and MKKs interact with non-phosphorylated JNKs, which

possess almost undetectable catalytic activity, we first confirmed that each compound binds to the ATP-binding site of non-phosphorylated JNK1. To do this, we developed a Time-Resolved Fluorescence Energy Transfer (TR-FRET) competition assay, that can be used to determine the affinities of ATP-binding site ligands, independent of kinase activation state (Figure 2A). This assay measures the ability of compounds to block TR-FRET between a europium-labeled antibody-bound to the N-terminal His₆-tag of JNK1-and a cyanine 5 (Cy5)-labeled, ATP-binding site ligand (Figures 2B, 2C). The inhibitory constants (K_i s) of our ten ATP-competitive inhibitors for non-phosphorylated JNK1 are shown in Figure 2D.

All ten inhibitors in our panel were capable of competitively displacing the Cy5-probe from the ATP-binding site of non-phosphorylated JNK1 (Figure 2D). Furthermore, we found that the IC₅₀ values for all ten ATP-competitive compounds for inhibition of activated phospho-JNK1 correlated well with their K_i s in the binding assay with non-phosphorylated JNK1. Most importantly, we found that all ten ATP-competitive inhibitors in our panel bind with high enough affinity to non-phosphorylated JNK1 that near quantitative JNK-inhibitor complexes can be generated for biochemical studies.

ATP-competitive inhibitors modulate JNK1's affinity for the D-domain of JIP1.

We next investigated whether ATP-binding site occupancy of JNK1 could allosterically modulate the interaction of its DRS with JIP1. To do this, we used a fluorescence polarization (FP) binding assay to measure the affinity of non-phosphorylated JNK1 and a 6-carboxytertamethylrhodamine(TMR)-peptide containing the D-domain of JIP1 (TMR-JIPtide). (Figures 3A, 3B).^{31, 38} Titration of non-phosphorylated *apo*-JNK1 against a fixed concentration of TMR-JIPtide provided a dissociation constant (K_D) for these two species of $0.89 \pm 0.09 \mu\text{M}$ (Figure 3C), which is consistent with prior studies.³⁸ We performed the same titration with ATP-bound JNK1 and found that this complex bound two-fold more tightly to JIPtide than *apo*-JNK1 (Figure 3D)

Next, we performed similar titrations with JNK1 complexed to each of the inhibitors in our panel, to determine how different modes of ATP-binding site occupancy affect JNK1's affinity for JIPtide. Titration of different JNK1-inhibitor complexes across a fixed concentration of TMR-JIPtide revealed that ATP-binding site occupancy can either strengthen or weaken JIPtide binding (Figure 3). Six out of the ten JNK1-inhibitor complexes tested showed a modest-but significant-increase in affinity for TMR-JIPtide, with the JNK1-1 complex demonstrating the largest overall enhancement (Figure 3E). We observed that three JNK1-inhibitor complexes demonstrated significantly diminished affinity for TMR-JIPtide relative to the *apo* or the ATP-bound forms of the kinase. Binding of inhibitor 2 caused the largest decrease in JIPtide affinity, with the JNK1-2 complex exhibiting an ~30-fold higher K_D for TMR-JIPtide compared to JNK1-ATP (Figure 3F). Overall, we found that the affinity of JNK1 for JIPtide can differ by ~50-fold, depending on the ligand occupying the ATP-binding site (Figure 3G).

ATP-competitive compounds modulate JIPtide binding to non-phosphorylated JNK2 and JNK3.

We performed similar binding assays with TMR-JIPtide and non-phosphorylated JNK2 and JNK3 to investigate any similarities or differences in how ATP-binding site occupancy influences JIPtide affinity amongst the JNKs. In general, we found that most of the inhibitors we tested have similar effects on all JNK isoforms (Table 1). For example, the **2**- and **6**-bound forms of all three JNKs demonstrated lower affinity for TMR-JIPtide relative to their *apo* forms. Furthermore, several inhibitors enhanced the interaction between TMR-JIPtide and all three JNK isoforms.

However, some inhibitors demonstrated divergent effects on the JNK isoforms. Consistent with the high sequence homology between JNK1 and JNK3 (92%), we observed the most similarity between these two isoforms in how ATP-binding site occupancy affected JIPtide binding affinity. JNK2 displayed the lowest affinity for TMR-JIPtide ($K_D = 7.9 \pm 0.8 \mu\text{M}$) and the most divergent behavior in how ATP-competitive ligands influenced its affinity for JIPtide. For example, compound **5** slightly increased JNK1's and JNK3's affinity for JIPtide but reduced the strength of the JNK2-JIPtide interaction by ~4 fold. Furthermore, JNK2 displayed the smallest fold differences between JIPtide's affinity for different ligand-bound complexes. These differences may reflect divergences in allosteric regulation between JNK family members.

Effects of ATP-binding site occupancy on JNK1's interaction with full-length JIP1.

We were curious whether the allosteric influence that our panel of inhibitors had on JNK1's interaction with JIPtide would also hold for full-length JIP1. To this end, we developed a JNK1 pull-down assay using immobilized, full-length JIP1 (Figure 4A). We used quantitative western blotting of retained JNK1 to determine the relative affinities of *apo*-JNK1 and different JNK1-inhibitor complexes for immobilized JIP1 (Figure S4). Consistent with the minimized JIPtide peptide behaving like the full-length protein scaffold, we observed that all ten inhibitors have a similar influence on JNK1's interaction with full-length JIP1 and JIPtide (Figure 4B). JNK1 complexed to compounds **1**, **3**, **5**, and **10** showed a significant increase in retained JNK1 relative to *apo*-JNK1, which reflects the higher binding affinities of these JNK1-inhibitor complexes for JIPtide. Furthermore, all four JNK1-inhibitor complexes that demonstrated reduced affinities for JIPtide also showed diminished retention by immobilized JIP1. Notably, the relative amounts of JNK1-**2**, JNK1-**4**, JNK1-**6**, and JNK1-**7** retained in the pull-down assay correlates with the binding affinities of these JNK1-inhibitor complexes for TMR-JIPtide. Given that we were unable to perform JNK1 titrations in the pull-down assay due to technical limitations, quantitative differences in affinities between different JNK1-inhibitor complexes for full-length JIP1 could not be determined. However our results demonstrate that TMR-JIPtide is a suitable surrogate for performing quantitative studies on the allosteric relationship between ATP-binding site occupancy and the DRSS of JNKs.

Assay for quantitative determination of JNK1 phospho-state.

MKK4 and MKK7 activate JNKs by phosphorylation of the TPY motif within their activation loops. Like JIP1, MKK4 and MKK7 use D-domains to interact with JNKs, but

whether these binding events are allosterically coupled to the ATP-binding sites of JNKs has not been explored.^{5, 22, 23} We were thus interested in using our inhibitors to determine how ATP-binding site occupancy affects the ability of MKK4 and MKK7 to phosphorylate the activation loops of JNKs. However, the mechanism by which MKK4 and MKK7 activate the JNKs is complex. While each of these MKKs have been shown to be capable of fully activating JNKs via dual phosphorylation of the TPY motif, this process is inefficient. In many circumstances, both MKKs are believed to be needed to achieve efficient dual phosphorylation. Therefore, prior to performing our studies, we developed a quantitative assay for measuring all phospho-forms of JNKs. To this end, we used Phos-tag sodium dodecyl sulfate polyacrylamide gel electrophoresis (Phos-tag SDS-PAGE), which allows phospho-forms of a protein to be separated and visualized based on the retarded migration of phosphorylated species by a polyacrylamide-immobilized manganese ion. (Figure 5A).^{39,40} We felt this assay would be ideal for our studies because it would allow us to quantify the ratio of non-, mono-, and dual-phosphorylated species.

We first determined whether Phos-tag SDS-PAGE could be used to separate JNK isoforms by activating JNK1 with a low or a high concentration of MKK4 (Figure 5B). Both activation conditions showed three bands when separated by Phos-tag SDS-PAGE and visualized with a total JNK antibody. For JNK1 activated with the lower concentration of MKK4, we observed a lower band that corresponds to non-phosphorylated JNK1, a middle band that is positive for phospho-Tyr but not dual phosphorylation, and a top band that is positive for dual-phosphorylated JNK1 (Figure 5B). Subjection of JNK1 to the higher concentration of MKK4 led to a significant increase in the top band, which corresponds to dual-phosphorylated JNK1, and diminution of the middle mono-phosphorylated JNK1 band. JNK1 activated with low or high concentrations of MKK7 produced similar distributions of JNK1 phospho-forms. However, under both activation regimes the middle band was not positive for phospho-Tyr, unlike MKK4-activated JNK1, which implies that this species is likely JNK1 mono-phosphorylated on Thr183. Our results are consistent with prior studies demonstrating MKK4's preference for Tyr185 and MKK7's preference for Thr183, even though both MKKs have been shown to be capable of achieving dual phosphorylation of JNKs in vitro.¹²

Having demonstrated that Phos-tag SDS-PAGE can separate all phospho-forms of JNK1, we next sought a more reproducible and quantitative method for determining JNK1 phospho-state. To this end, we created TMR-JNK1 (Figure 5C), using the transpeptidase Sortase A to transfer TMR-LPTY-from a TMR-labeled, substrate peptide—to the free N-terminus of JNK1 (Figures 5D).⁴¹ This method allowed us to separate the JNK1 phospho-forms using Phos-tag SDS-PAGE, and directly visualize them using fluorescence imaging of the TMR-JNK1. We observed that high concentrations of MKK4 or MKK7 led to similar ratios of phosphostates for TMR-JNK1 and unlabeled-JNK1, demonstrating that the fluorescently-labeled construct behaves like untagged JNK1 (Figure 5E).

Kinetic analysis of JNK1 activation.

Prior to performing studies on how ATP-binding site occupancy affects the activation of JNKs, we used our new assay to provide the first quantitative look at the generation of

mono- and dual-phosphorylated JNK1 by MKK4 and MKK7 (Figure 6A). We began by conducting titrations with MKK4 or MKK7 to determine how generation of mono- and dual-phosphorylated TMR-JNK1 correlates to the activities of these enzymes (Figure 6B). We observed a graded increase in the amount of mono-phosphorylated JNK1 generated as MKK4 concentrations were elevated, until a sufficient amount of mono-phosphorylated JNK1 was formed that higher concentrations of MKK4 converted to the dual-phosphorylated species. We saw a similar effect with the MKK7 titration, except the transitions from the non-phosphorylated to the mono-phosphorylated species and from the mono-phosphorylated to the dual-phosphorylated species were more stepwise than graded. Thus, MKK4 and MKK7 show distinct behaviors in the amount of activity required to generate different phospho-forms of JNK1.

We then conducted time courses for TMR-JNK1 activation using three MKK concentrations that span the activation regimes we observed at the single, one hour timepoint in Figure 6B. We found that the lowest concentration of MKK4 led to a near linear increase in mono-phosphorylated TMR-JNK1 over time, with a similar linear increase observed for MKK7 as well (Figures 6C, 6D). Activation of TMR-JNK1 with a 30-fold higher concentration of MKK4 led to rapid formation—within five minutes—of mono-phosphorylated JNK1 that minimally increased over time, and barely detectable dual-phosphorylated JNK1 at the last timepoint (Figure 6E). An almost identical kinetic behavior was observed for activation of TMR-JNK1 with an intermediate concentration of MKK7 (Figure 6F). Interestingly, co-activation of TMR-JNK1 with both MKK4 and MKK7 at the same intermediate concentrations led to rapid—within 30 seconds—generation of mono- and dual-phosphorylated JNK1 that was converted predominately to the dually phosphorylated species over time (Figure 6G). This observation is consistent with prior studies showing that MKK4 and MKK7 likely work synergistically to achieve efficient full (dual) activation of JNK in cells.^{12, 13} The highest concentration of MKK4 led mainly to mono-phosphorylated TMR-JNK1 at the first timepoint tested (30 seconds), followed by an almost linear conversion of the mono-phosphorylated species to dual over 20 min (Figure 6H). Again, we observed an almost identical kinetic behavior for activation of TMR-JNK1 by the highest concentration of MKK7 (Figure 6I). Thus, although MKK4 and MKK7 demonstrate differences in the amount of activity required to transition between different JNK1 phospho-forms (Figure 6B) and in the identity of the mono-phosphorylated species that they generate (Figure 5B), their kinetic behavior in the activation of JNK1 appears to be quite similar.

ATP-competitive inhibitors can enhance or diminish JNK1 activation loop phosphorylation by MKK7 and MKK4.

Next, we sought to determine how varying the occupancy of JNK1's ATP-binding site influenced the ability of MKK7 to generate mono- and dual-phosphorylated JNK1 (Figure 7A). First, we measured how much mono- and dual-phosphorylated JNK1 were produced when different JNK1-inhibitor complexes were activated with a high concentration of MKK7, relative to JNK1 that was not bound to an ATP-competitive inhibitor (Figure 7B). Using this activation regime, we observed that the dual-phosphorylation of JNK1's activation loop by MKK7 is either enhanced, diminished, or unaffected when it is bound to ATP-competitive inhibitors. Inhibitors **2**, **4**, **5**, **7**, and **10** had only a small effect on the

amount of mono- and dual-phosphorylated JNK1 generated, while JNK1 bound to **1**, **3**, **8**, or **9** was more readily converted to the dual-phosphorylated species relative to JNK1 in the absence of an ATP-competitive inhibitor. Notably, **1**, **3**, **8**, and **9** also increase the affinity of the JIP1 scaffold protein for JNK1 (Figures 3C, 4B), suggesting that MKK7's interaction with JNK1 may be strengthened by a similar mechanism.

Our JNK1 activation results at a high concentration of MKK7 demonstrated that compounds **1**, **3**, **8**, and **9** enhanced generation of the dual-phosphorylated species at the expense of the mono-phosphorylated form. Therefore, we next measured how efficiently the activation loops of different JNK1-inhibitor complexes were phosphorylated by a concentration of MKK7 that does not generate the dual-phosphorylated form (Figure 6B). Under this activation regime, we found that compounds **1**, **3**, and **8** had little or no effect on MKK7's ability to generate mono-phosphorylated JNK1 (Figure 7C). In contrast, compound **9** dramatically decreased the amount of mono-phosphorylated JNK1 generated, despite promoting the production of dual-phosphorylated JNK1 at a higher MKK7 concentration. These results suggest that unphosphorylated JNK1's interaction with **1**, **3**, **8**, or **9** does not make it a better substrate for MKK7 relative to JNK1 in the absence of an ATP-competitive inhibitor. However, once these inhibitor-bound JNK1 complexes become mono-phosphorylated, they are more readily converted to the dual-phosphorylated species by MKK7. JNK1 bound to compound **9** is the most striking example of this trend; the JNK1-**9** complex is a poor substrate for MKK7, but the mono-phosphorylated JNK1-**9** complex is efficiently converted to the dual-phosphorylated species.

ATP-competitive inhibitors can enhance or diminish phosphorylation of JNK1 by MKK4.

We next determined whether MKK4's ability to generate mono- and dual-phosphorylated JNK1 was similarly influenced by JNK1's interaction with ATP-competitive inhibitors (Figure 8A). Like with MKK7, we first measured how much mono- and dual-phosphorylated JNK1 were produced when different JNK1-inhibitor complexes were activated with a high concentration of MKK4, relative to JNK1 that was not bound to an ATP-competitive inhibitor. Using this activation regime, we found that most inhibitor-bound JNK1 complexes were similarly acted upon by MKK4 and MKK7 (Figure 8B). For example, inhibitors **2**, **4**, **5**, and **7** had only a small effect on the amount of mono- and dual-phosphorylated JNK1 generated by MKK4, while JNK1 bound to **3**, **8**, or **9** was more readily converted to the dual-phosphorylated species relative to *apo*-JNK.1. Furthermore, the JNK1-**6** complex was a poor substrate for MKK4, like it was for MKK7 (Figure 7B). The only major difference between MKK4 and MKK7 was their relative abilities to generate mono- and dual-phosphorylated species of the JNK1-**1** complex. JNK1's interaction with **1** makes it a better substrate for MKK7, while this ligand makes it an inferior substrate for MKK4.

Like with MKK7, we next measured how efficiently the activation loops of different JNK1-inhibitor complexes were phosphorylated by a concentration of MKK4 that does not generate the dual-phosphorylated form (Figure 6B). Under this activation regime, we found that MKK4 behaved similarly to MKK7 with some small differences (Figure 8C). In contrast to MKK7, compounds **3** and **8** slightly increased the amount of mono-

phosphorylated JNK1 produced by MKK4. However, consistent with the unique behavior observed for the JNK1-9 complex with MKK7, JNK1's interaction with 9 dramatically decreased the amount of mono-phosphorylated JNK1 generated by the lower concentration of MKK4, despite promoting the production of dual-phosphorylated JNK1 at the higher MKK4 concentration. Furthermore, the JNK1-6 complex appears to be a poor substrate for low and high concentrations of MKK4, like it is for MKK7. One possible mechanism for 1 and 6 to reduce the activation loop phosphorylation of JNK1 is through direct inhibition of MKK4. To exclude this possibility, we conducted inhibition assays with both compounds using an alternative MKK4 substrate, the MAPK p38 α (Figure S5)⁴². Consistent with both 1 and 6 diminishing activation loop phosphorylation through their interaction with JNK1 and not MKK4, neither compound reduced the ability of MKK4 to phosphorylate p38 α . Therefore, JNK1's interaction with ATP-competitive inhibitors can make it either a less or more efficient substrate for MKK4.

One possible explanation for why some inhibitors, like 3 and 8, make JNK1 a better substrate for MKK4 and MKK7 is that these inhibitor-bound complexes have higher affinities for MKKs, like they do for JIP1. To test this possibility, we performed pull-down assays with *apo*-JNK1 and inhibitor-bound complexes using MKK4 as an immobilized bait (Figure 8D). We observed that both the JNK1-3 and JNK1-8 complexes, which are more efficiently converted to the dual phosphorylated form by both MKK4 and MKK7, were pulled down more efficiently by MKK4 than *apo*-JNK.1. Therefore, it appears that JNK1's interactions with upstream activators can be allosterically modulated through its ATP-binding site like its interactions with scaffold proteins. Because ATP-competitive inhibitors that modulate the phosphorylation of JNK1 by MKK4 and MKK7 promote dual over mono-phosphorylation, the enhanced affinity we observe most likely influences processivity, instead of the first phosphorylation event.

Previous structural studies suggested that D-domain interactions with the DRSs of JNKs allosterically influence the conformation of JNK ATP-binding sites. However, the full extent of the allosteric communication between the DRSs and the ATP-binding sites of JNKs has not been previously explored. Here, we systematically investigated the effects of ten ATP-competitive JNK inhibitors on DRS interactions. First, we investigated how ATP-binding site occupancy affected binding of a peptide containing the D-domain of JIP1 (JIPTide). Using a quantitative fluorescence polarization binding assay, we demonstrated that the interactions of JNKs with JIPTide can either be strengthened or weakened through the ATP-binding site. In general, we found that most of the ATP-competitive inhibitors we tested had similar effects on all JNK isoforms. We observed a high amount of similarity between JNK1 and JNK3 but noted some differences in how JNK2's affinity for JIPTide was modulated. These results are consistent with the higher sequence homology of JNK1 and JNK3, compared to JNK2, and likely indicate that there are differences in allostery across the JNK family. After determining the effects of JNK ATP-binding site occupancy on JIPTide binding, we validated that these effects were similar for the full-length scaffold protein with a pull-down assay using immobilized JIP1. Together, these results demonstrated that JIPTide is a suitable surrogate of full-length JIP1 for studies exploring allosteric communication between

the DRSs and ATP-binding sites of JNKs and that different inhibitor-bound JNK complexes can have large differences in their affinities for scaffold proteins.

We then explored how the ATP-binding site occupancy of JNKs influenced the abilities of MKK4 and MKK7 to phosphorylate their activation loops. To do this, we developed a Phos-tag SDS-PAGE based assay that allowed us to separate the phospho-forms of fluorophore-labeled JNK1. This technique allowed us to ratio-metrically quantify the phosphorylation of JNK1's activation loop. We first used this new assay to conduct a quantitative kinetic study of MKK4's and MKK7's activation of JNK1. These kinetic analyses validated previous studies showing that MKK4 and MKK7 are likely to work synergistically to achieve efficient dual phosphorylation in cells. We then investigated how the interactions of our ten inhibitors with the ATP-binding site of JNK1 influenced the ability of MKK4 and MKK7 to phosphorylate the activation loop of JNK1. These studies showed that different JNK1-inhibitor complexes were significantly better or worse substrates for MKK4 and MKK7. Consistent with certain inhibitors generally promoting higher affinity interactions between D-domain binding partners and the DRSs of JNKs, many of the inhibitors that increased JNK1's affinity for JIP1 led to enhanced activation loop phosphorylation by MKK4 and MKK7. Our results further validate the existence of allostery between the ATP-binding sites of JNKs and their distal D-recruitment sites.

This study adds the JNKs to the growing list of kinases whose interactions with protein binding partners can be divergently modulated through allostery with ATP-competitive inhibitors.^{37, 43-50} How divergently modulating interactions with MKK4, MKK7, and JIP1 affects the behavior of JNKs in cells will need to be empirically determined but several predictions can be made. It would be expected that inhibitors that promote dual phosphorylation of JNK1 by MKK4 and MKK7 would result in hyper-activation of JNK signaling under drug wash-out conditions, which occurs during most *in vivo* drug treatments. Furthermore, in most cellular environments, where JNKs are expressed in excess of JIP1, isoform-selective inhibitors that promote the interaction of inhibitor-bound JNKs with JIP1 would be predicted to prevent scaffold-mediated activation of uninhibited JNK isoforms. Finally, based on the bidirectional nature of allosteric networks, it is likely that JIP1-bound JNKs will be more or less sensitive to certain ATP-competitive inhibitors than their uncomplexed forms. JNKs have been shown to interact with the D-domains of numerous scaffolds, downstream substrates, and upstream activators via their DRSs, therefore, ATP-competitive inhibitors could significantly alter their interactions with a number of cellular binding partners.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

(JNKs)	c-Jun NH ₂ -terminal kinases
(JIP)	JNK-interacting protein
(JIPtide)	11-mer D-domain peptide of JIP1
(DRS)	D-recruitment site
(MAPK)	mitogen activated protein kinase
(MKK)	MAPK kinase
(ATP)	adenosine triphosphate
(EDTA)	ethylenediaminetetraacetic acid
(HEPES)	N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid
(DTT)	dithiothreitol
(BSA)	bovine serum albumin
(EGTA)	ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
(Cy5)	cyanine 5
(TMR)	6-carboxytertamethylrhodamine
(FP)	fluorescence polarization
(Phos-tag SDS-PAGE)	Phos-tag sodium dodecyl sulfate polyacrylamide gel electrophoresis
(Ni-NTA)	Ni ²⁺ -nitrilotriacetate
(BME)	β-mercaptoethanol
(Tris)	tris(hydroxymethyl)aminomethane
(DMSO)	dimethyl sulfoxide
(NMR)	Nuclear Magnetic Resonance
(MS)	Mass Spectrometry

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Figure 1: Allosteric communication between the DRSs and ATP-binding sites of JNKs.

(A) Structure of JNK1 bound to JIPtide (blue, PDB ID: 4E73) or the D-domain of MKK7 (green, PDB ID: 4UX9). The DRS and ATP-binding site of JNK1 are circled. (B) The ten ATP-competitive JNK compounds that were used in this study and their IC₅₀ values for JNK1 and JNK2 in a TR-FRET activity assay.

Figure 2: A competitive binding assay for determining the affinities of JNK inhibitors for non-phosphorylated JNKs.

(A) Schematic of the TR-FRET-based competition binding assay. (B) Structure of the Cy5-labeled, ATP-binding site probe (Cy5-Probe). (C) Titration curve of the Cy5-probe's binding interaction with non-phosphorylated JNK1. (D) Inhibitory constants (K_i) of compounds 1-10 for non-phosphorylated JNK1. Values shown are mean $\pm$ SEM (n=3).

Figure 3: Influence of JNK ATP-binding site occupancy on JIPTide affinity.

(A) Two modes of how ATP-competitive inhibitors can modulate the affinity of JNKs for TMR-JIPTide. Disruptors reduce the affinity of JNKs for TMR-JIPTide by allosterically stabilizing a docking site conformation with reduced complementarity to TMR-JIPTide. Enhancers increase the affinity of JNKs for TMR-JIPTide by allosterically stabilizing a docking site conformation with increased complementarity to JIPTide. (B) Schematic of how JNK-enhancer and JNK-disruptor complexes behave in the TMR-JIPTide FP assay. (C) K_{DS} of JIPTide for apo-JNK1 and JNK1-inhibitor complexes. Values shown are mean $\pm$ SEM ($n=3$). (D-G) Titration curves of apo-JNK1 and JNK1-inhibitor complexes against TMR-JIPTide.

Figure 4: Effects of ATP-binding site occupancy on JNK1's interaction with full-length JIP1. (A) Schematic of the pull-down assay. (B) Full-length Flag-JIP1 (0.6 μ M) was immobilized and then incubated with either 200 nM JNK1 (left) or 500 nM JNK1 (right), $\pm$ 10 μ M ATP-competitive inhibitor. The JNK1-JIP1 complexes were washed and remaining JNK1-JIP1 was eluted from the beads. JIP1 and JNK1 were detected by western blotting for total JNK (JNK1) and Flag (JIP1). Values shown are mean $\pm$ SEM (n=3). Statistical significance was determined by a two-tailed, unpaired t-test. See Figure S4 for JNK1 western blot linearity curves, which allow quantitative comparisons.

Figure 5: Assay for quantitative determination of JNK1 phospho-state.

(A) Phos-tag SDS-PAGE was used to separate and quantify non-, mono-, and dual-phosphorylated JNK1. (B) JNK1 (200 nM) was activated with either high (900 nM) or low (30 nM) MKK4 or MKK7 concentrations, and the reaction was separated by Phos-tag SDS-PAGE. Western blotting for total JNK, pT183/pY185 JNK (dual phosphorylation), and pTyr was used to confirm the identity of the JNK1 phospho-forms. (C) Structure of TMR-labeled JNK1. (D) Sortase A method for labeling the N-terminus of JNK1 with TMR (for gels showing creation and purification of TMR-JNK1, see Supplemental Figure 3). (E) TMR-JNK1 (200 nM) was activated with either 900 nM MKK4 or MKK7, separated by Phos-tag SDS-PAGE, and imaged using fluorescence scanning.

Figure 6: Quantitative analyses of TMR-JNK1 phosphorylation by MKK4 and MKK7.

(A) TMR-JNK1 was activated under varying conditions and resulting phospho-forms were quantified with fluorescence scanning following Phos-tag SDS-PAGE. (B) Varying concentrations of MKK4 or MKK7 were used to activate TMR-JNK1 for one hour and JNK1 phospho-forms were resolved with Phos-tag SDS-PAGE. (C-H) Kinetic analyses of TMR-JNK1 activation under different MKK4 and MKK7 concentration regimes. Values shown are mean $\pm$ SEM (n=3). (C) TMR-JNK1 activation by 1 nM MKK4. (D) TMR-JNK1 activation by 1 nM MKK7. (E) TMR-JNK1 activation by 30 nM MKK4. (F) TMR-JNK1 activation by 30 nM MKK7. (G) TMR-JNK1 activation by 30 nM MKK4 and 30 nM MKK7. (H) TMR-JNK1 activation by 900 nM MKK4. (I) TMR-JNK1 activation by 900 nM MKK7.

Figure 8: Quantitative assessment of how ATP-competitive inhibitors affect JNK1 phosphorylation by MKK4.

(A) Assay to assess how the ATP-binding site occupancy of JNK1 influences its activation by MKK4. (B) TMR-JNK1 was incubated with ATP and different ATP-competitive inhibitors, followed by the addition of MKK4 (250 nM). After one hour of incubation, JNK1 phospho-forms were separated by Phos-tag SDS-PAGE, detected, and quantified using fluorescence scanning. Values shown are mean $\pm$ SEM (n=3). Statistical significance was determined by a two-tailed, unpaired t-test. (C) Experiments conducted as in (B), except with 0.25 nM MKK4. (D) Schematic of the JNK1-MKK4 pull-down assay (left) and quantification of TMR-JNK1 pull-down in the absence or presence of compounds **3** and **8** (right).

Table 1:

Fluorescence polarization binding assay.

Compound	JNK/TMR-JIPTide K_{DS} (μ M) $\pm$ 30 μ M Compound		
	JNK1	JNK2	JNK3
DMSO	0.89 $\pm$ 0.09	7.9 $\pm$ 0.8	1.9 $\pm$ 0.2
1	0.23 $\pm$ 0.03	4.6 $\pm$ 0.4	0.48 $\pm$ 0.01
2	14 $\pm$ 1	17 $\pm$ 2	12 $\pm$ 2
3	0.25 $\pm$ 0.01	3.0 $\pm$ 0.2	0.64 $\pm$ 0.08
4	1.5 $\pm$ 0.1	6.0 $\pm$ 0.8	2.8 $\pm$ 0.2
5	0.68 $\pm$ 0.09	37 $\pm$ 8	1.7 $\pm$ 0.2
6	3.2 $\pm$ 0.4	15 $\pm$ 1	4.6 $\pm$ 0.2
10	0.44 $\pm$ 0.09	5.1 $\pm$ 0.2	1.5 $\pm$ 0.1