Accepted Manuscript

Identification and characterization of bi-thiazole-2,2'-diamines as kinase inhibitory scaffolds

Kevin R.W. Ngoei, Dominic C.H. Ng, Paul R. Gooley, David P. Fairlie, Martin J. Stoermer, Marie A. Bogoyevitch

PII: S1570-9639(13)00064-2
DOI: doi: [10.1016/j.bbapap.2013.02.001](https://doi.org/10.1016/j.bbapap.2013.02.001)
Reference: BBAPAP 39014

To appear in: *BBA - Proteins and Proteomics*

Received date: 16 August 2012
Revised date: 30 January 2013
Accepted date: 3 February 2013

Please cite this article as: Kevin R.W. Ngoei, Dominic C.H. Ng, Paul R. Gooley, David P. Fairlie, Martin J. Stoermer, Marie A. Bogoyevitch, Identification and characterization of bi-thiazole-2,2'-diamines as kinase inhibitory scaffolds, *BBA - Proteins and Proteomics* (2013), doi: [10.1016/j.bbapap.2013.02.001](https://doi.org/10.1016/j.bbapap.2013.02.001)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**Identification and characterization of bi-thiazole-2,2'-diamines
as kinase inhibitory scaffolds**

**Kevin R.W. Ngoei¹, Dominic C.H. Ng¹, Paul R. Gooley¹, David P. Fairlie²,
Martin J. Stoermer^{2*} and Marie A. Bogoyevitch^{1*}**

¹ Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville, Victoria 3010, Australia.

² Division of Chemistry and Structural Biology, Institute for Molecular Bioscience, The University of Queensland, Brisbane, Queensland 4072, Australia

Running Title:

Protein kinase inhibition by bi-thiazole-2,2'-diamines

*Address correspondence to:

Marie A. Bogoyevitch. Phone: +61 3 8344 2289 Fax: +61 3 9348 1421.

Email: marieb@unimelb.edu.au.

Martin J. Stoermer. Phone: +61 7 3346 2991 Fax: +61 7 9348 2101.

Email: m.stoermer@imb.uq.edu.au

Abstract (Word Count: 249)

Based on bioinformatics interrogation of the genome, >500 mammalian protein kinases can be clustered within seven different groups. Of these kinases, the mitogen-activated protein kinase (MAPK) family forms part of the CMGC group of serine/threonine kinases that includes extracellular signal regulated kinases (ERKs), cJun N-terminal Kinases (JNKs), and p38 MAPKs. With the JNKs considered attractive targets in the treatment of pathologies including diabetes and stroke, efforts have been directed to the discovery of new JNK inhibitory molecules that can be further developed as new therapeutics. Capitalizing on our biochemical understanding of JNK, we performed *in silico* screens of commercially available chemical databases to identify JNK1-interacting compounds and tested their *in vitro* JNK inhibitory activity. With *in vitro* and cell culture studies, we showed that the compound, 4'-methyl-N²-3-pyridinyl-4,5'-bi-1,3-thiazole-2,2'-diamine (JNK Docking (JD) compound 123, but not the related compound (4'-methyl-N~2~-(6-methyl-2-pyridinyl)-4,5'-bi-1,3-thiazole-2,2'-diamine (JD124), inhibited JNK1 activity towards a range of substrates. Molecular docking, saturation transfer difference NMR experiments and enzyme kinetic analyses

revealed both ATP- and substrate-competitive inhibition of JNK by JD123. In characterizing JD123 further, we noted its ATP-competitive inhibition of the related p38- γ MAPK, but not ERK1, ERK2, or p38- α , p38- β or p38- δ . Further screening of a broad panel of kinases using 10 μ M JD123, identified inhibition of kinases including Protein Kinase B β (PKB β /Akt β). Appropriately modified thiazole diamines, as typified by JD123, thus provide a new chemical scaffold for development of inhibitors for the JNK and p38- γ MAPKs as well as other kinases that are also potential therapeutic targets such as PKB β /Akt β .

Abbreviations

MAPK, mitogen-activated protein kinase; JD, JNK Docking; ERK, extracellular signal signal-regulated kinase; JNK, c-Jun N-terminal Kinase; MKK, MAPK Kinase; GST, glutathione S-transferase; GSH, glutathione; JIP, JNK-interacting protein; DCX, doublecortin X; EGFR, epidermal growth factor receptor; PKB/Akt, Protein Kinase B; STD NMR, saturation transfer difference nuclear magnetic resonance; AMP-PNP, adenosine 5'-(β,γ -imido)triphosphate; DMSO, dimethyl sulfoxide; MEF, murine embryonic fibroblast; PVDF, polyvinylidene fluoride.

1. Introduction

Protein phosphorylation, as mediated by the ATP γ -phosphate transferase activities of protein kinases, is a critical regulator in a diversity of cellular events [1-3]. Whilst protein kinases can be broadly classified as Ser/Thr kinases, Tyr kinases or dual-specificity (Ser/Thr and Tyr) kinases based on their substrate preference, genome-wide bioinformatic analyses using conserved kinase sequence motifs have revealed a kinase superfamily and the relationships between groupings of protein kinases [4-7]. Based on these “kinome” analyses, >500 mammalian protein kinases broadly grouped within seven different clusters have been predicted [6, 7]. The mammalian mitogen-activated protein kinases (MAPKs), part of the larger CMGC grouping of serine/threonine kinases, are a well-studied family that includes the extracellular signal regulated kinases (ERKs), the cJun N-terminal Kinases (JNKs) and the p38 MAPKs, p38- α , - β , - γ and - δ . For the ERKs, significant roles in growth factor-mediated events have been defined, whereas roles for JNKs and p38 MAPKs have been evaluated primarily following stress and/or cytokine stimulation [8].

The JNKs were the initial focus of our present study. Three mammalian JNK-encoding genes have been identified (*jnk1*, *jnk2* and *jnk3*) [9, 10] with alternative splicing generating JNK isoforms varying between 46 and 55 kDa [11, 12]. Recent reports have implicated actions of JNKs in various diseases such as stroke [13, 14], neurodegenerative disorders including Alzheimer’s Disease [15, 16] and Parkinson’s Disease [17], type-2 diabetes [18, 19], inflammation [20-22] and cancer [23, 24]. Therefore, significant attention has been directed towards the identification of JNK inhibitory molecules.

The discovery process for JNK inhibitors has largely focused on the screening of proprietary chemical libraries for their direct inhibition of the kinase activity of JNK *in vitro* (see review [25]). This process has revealed small molecule ATP-competitive inhibitors of JNK (e.g. SP600125 [26], AS601245 [27], and others [28, 29]; see reviews [25, 30]). In addition, a small peptide derived from the JNK pathway scaffolding protein, JNK-interacting protein 1 (JIP1) [31], has revealed the potential for a new group of ATP-noncompetitive JNK inhibitors. Indeed, this minimal inhibitory peptide derived from JIP1 interacts with the substrate docking site of JNK [31-33], and so competes with protein substrates for binding to JNK. Whilst this peptide has provided biochemical insights into the mechanisms used by JNK to recruit its specific downstream substrate proteins, further use of cell-permeable versions of these JNK inhibitory peptides has generated considerable interest with the observed beneficial effects to improve neuronal survival following stroke insult to the brain [13], to improve symptoms in diabetes [34] and to provide protection in inflammatory bowel disease [35].

In approaching new methodologies to develop protein kinases inhibitors, we have undertaken a virtual screen to identify small molecules predicted to occupy the substrate-docking/JIP peptide interaction site of JNK1 [32]. Here, we report the results of this screen, showing the JNK inhibitory properties of a group of bi-thiazole-2,2'-diamines. Interestingly our kinetic analysis showed these to act as dual-inhibitors of JNK, competing with both protein substrate and ATP. Our further biochemical characterization of a promising lead compound, 4'-methyl-N²-3-pyridinyl-4,5'-bi-1,3-thiazole-2,2'-diamine (coded here as JNK Docking (JD) compound 123), revealed the inhibition of p38- γ MAPK in an ATP-competitive manner as well as the inhibition of other less related kinases, including Protein Kinase B β (PKB β /Akt β). Whilst strict kinase specificity has been a goal of previous kinase inhibitor discovery programmes, the potential benefits of targeting multiple kinases for inhibition and the discovery of new

scaffolds that generate inhibitors of greater diversity, has also been discussed [36, 37]. Our results highlight that appropriately modified thiazole diamines can provide a new chemical scaffold for development of small molecule inhibitors directed towards the JNKs and p38- γ as well as other kinases including PKB β /Akt β .

2. Experimental Procedures

2.1. Virtual Screening Approaches and Molecular Docking Analyses

For the 11 amino acid JNK inhibitory peptide known as TIJIP, four residues have been described as critical for JNK inhibition as denoted by underlining in the amino acid sequence RPKRPTTLNLF [31, 33]. These residues correspond to ¹⁵⁶Arg, ¹⁵⁷Pro, ¹⁶⁰Leu and ¹⁶²Leu within the full length JIP1 sequence and largely agree with the recognized JNK binding-consensus, (Arg/Lys)-X₁₋₄- ϕ -X- ϕ , where ϕ represents hydrophobic residues [11, 38, 39]. The structure of JNK1 α 1 bound to the JIP1 peptide is available in the public domain (Protein Data Bank (PDB): 1ukh) [32]. We divided this docking site of JNK1 into two regions and focused on the region centred on ¹⁵⁶Arg-¹⁵⁷Pro (referred to throughout as the “RP site”). We further prepared this structure for docking by excising the JIP1 peptide and then adjusting the protonation states of its amino acids to their states at pH 7.0 using InsightII (Accelrys). In all subsequent docking analyses, the RP site was targeted via a coarse-grained hierarchical docking protocol described in the following paragraphs.

The Druglike, Leadlike and Fragment-like chemical databases were obtained from ZINC (version 8) and used without further filtering. The databases were divided into 1 million

compound subsets. These were docked using GOLD (v.3.2) [40] with the “GOLD-library screening” setting. The top 5000 ranked molecules from each run were retained and molecules identified in this first pass were then combined and re-docked using the default GOLD settings with 10 independent dockings for each molecule. The highest-ranked binding pose for each of the 500 top-ranking molecules was then visually evaluated to identify diverse candidate ligands.

The list of 500 candidates was further refined for subsequent purchase and *in vitro* testing based on properties of the RP site. As the RP binding site contains a shallow hydrophobic pocket and a series of acidic (³²⁹Glu, ³²⁶Asp) and polar (¹³⁰Tyr, ¹³³Tyr) residues that interact with the Arg side-chain, priority was given to a range of chemotypes that possessed a small ring system and a potentially either charged or hydrogen bond donating substituent in place of Arg. These were typified by the aryl substituted aminothiazole series of compounds. In addition, we further directed our choices by solubility characteristics of the identified compounds, selecting to purchase and test those compounds considered to be less hydrophobic (cLogP < 5) and therefore more soluble under our *in vitro* kinase assay conditions.

2.2. Preparation of active JNK1 α 1 and JNK2 α 2

Full-length JNK1 α 1 or JNK2 α 2 cDNAs containing a 5' glutathione S-transferase (GST) were subcloned into the *Xba*I and *Xho*I sites in pBACPAK9 vector (Clontech) to create the plasmids, pBACPAK9-GST-JNK1 pBACPAK9-GST-JNK2, respectively. In addition, full length but untagged MAPK Kinase 4 (MKK4) and MKK7 constructs were subcloned to create pBACPAK9-MKK4 and pBACPAK9-MKK7. Each of these plasmids was individually

co-transfected with BACPAK6 baculoviral DNA into *Spodoptera frugiperda* 9 (Sf9) insect cells to generate recombinant JNK1, JNK2, MKK4 and MKK7 baculoviruses according to manufacturer's protocol (Clontech) [41]. Western blotting was conducted using anti-JNK antibodies (BD Biosciences), anti-MKK4 or anti-MKK7 antibodies (Santa Cruz) to determine protein expression post-infection, and each recombinant baculovirus was subsequently enriched through plaque purification assays.

Cultures (1 L) of Sf9 cells (1×10^6 cells/mL) were infected with recombinant JNK1 α 1 or JNK2 α 2 baculovirus. For production of active form of the JNKs, each culture was further infected with baculovirus encoding the upstream kinases, MKK4 and MKK7. Each virus was used at a multiplicity of infection of 1.0. After 3 days post-infection, cells were harvested by centrifugation for further purification of JNK using standard GSH-sepharose affinity followed by ion exchange chromatography. The GST was removed by PreScission Protease (GE Healthcare) cleavage prior to further use.

2.3. JNK3 and other related MAPKs

Purified recombinant kinases, JNK3 α 1, ERK1, ERK2 and p38- γ , were purchased from Upstate Cell Signalling Solutions/Millipore.

2.4. Expression and Preparation of Recombinant Substrate Proteins

The JNK substrates (cJun (1-135), ATF2 (19-96), Elk1 (307-428), DCX (1-366)) were prepared from *Escherichia coli* as GST-fusion proteins [41].

2.5 JNK Inhibitory Compounds

Compounds identified in our virtual screening were purchased from ChemBridge and tested as JNK inhibitors. For ease of reference, compounds were coded with the JNK-docking (JD) nomenclature (from JD100-128, see Supplementary Table 1 for complete listing of all compounds tested in these series). The positive control JNK inhibitory peptide derived from the JIP1 scaffold protein, TIJIP [31], was synthesized (purity >80% by HPLC and mass spectrometry) by Proteomics International (Perth, Australia). All chemicals and the TIJIP peptide were dissolved in dimethyl sulphoxide (DMSO) at room temperature with brief vortexing and remained soluble under subsequent assay conditions.

2.6. *In vitro* Activity Assays and Kinetic Analyses

The activity of JNK towards recombinant GST-fusion proteins of different substrates (10 µg of either the transcription factors: cJun (1-135), ATF2 (19-96), or Elk1 (307-428) or the microtubule regulatory protein DCX (full length, 1-366), was examined in the presence of the JD100 series chemicals (initial testing at 100 µM, and for characterization of JD123 at varying concentrations as indicated) in a kinase reaction buffer (20 mM HEPES, 20 mM β-glycerophosphate, pH 7.6, supplemented with 20 mM MgCl₂, 25 µM sodium orthovanadate and 100 µM dithiothreitol). After preincubation (5 min) of JNK with its protein substrate and the chemical inhibitors, each kinase reaction was initiated by the addition of ATP (5 µM ATP, 1 µCi of [γ-³²P] ATP) followed by incubation for 20 min at 30°C. Phosphorylated proteins were separated by SDS-PAGE (12% gels), and ³²P incorporation visualised using autoradiography then quantitated by Cerenkov counting. Additional control assays included the characterized JNK inhibitory peptide (TIJIP, 2 µM [31]). All assays were repeated on

three independent occasions and statistical differences in inhibition were determined by Student's paired t-test.

Assays with the related MAPKs, ERK1, ERK2 and p38- γ followed similar protocols. Specifically, ERK1, ERK2 or p38- γ (2 ng each) was incubated with recombinant protein substrate (GST-Elk1 for ERKs, GST-ATF2 for p38- γ), each kinase reaction initiated by the addition of ATP (5 μ M ATP, 1 μ Ci of [γ - 32 P] ATP). To explore the broader kinase inhibitory actions of JD123 and JD124, these compounds were tested by the kinase inhibitor profiling service at the International Centre for Kinase Profiling (www.kinase-screen.mrc.ac.uk; formerly the Division of Signal Transduction Therapy, University of Dundee) under their standard kinase activity assay conditions (including 5, 20 or 50 μ M ATP, as chosen to be at or below the K_m for ATP for each kinase [42]). Parallel testing of positive control compounds, including the reported JNK inhibitor SP600125 [26], and replicates of all assays were performed routinely to ensure reproducibility within and between assays. Quality control estimates included the logIC₅₀ for inhibition falling within a half log unit of the rolling mean of the kinase assays.

To assess the mechanisms of p38- γ and JNK inhibition by JD123, the concentrations of substrates and ATP were varied along with the concentration of the chemical inhibitors (10-40 μ M JD123, 0.8-8.1 μ M cJun for JNK or 0.8-8.1 μ M ATF2 for p38- γ , 2-20 μ M ATP). Data was analyzed by double-reciprocal plot ($1/v$ (min/pmol) vs $1/[GST-cJun (1-135)]$ (μ M⁻¹) or $1/[ATP]$ (μ M⁻¹)) and *linear regression analysis* was performed.

Additional activity assays were conducted to assess the mechanism of JD123-mediated JNK inhibition. Thus, JNK1 activity was assessed towards Epidermal Growth Factor Receptor

(EGFR)-based peptide substrate (sequence: KRELVEPLTPSGEA; Peptide Technologies, Bio21 Institute, Melbourne, Victoria) [43]. Following preincubation (5 min) active JNK1 α 1 with the peptide substrate (8 μ M) and 10-100 μ M JD123 (including 2 μ M TIJIP or 10 μ M ATP competitive inhibitor JNK inhibitor VIII (N-(4-Amino-5-cyano-6-ethoxypyridin-2-yl)-(2,5-dimethoxyphenyl)acetamide [28], Calbiochem), each kinase reaction was initiated by the addition of ATP (5 μ M ATP, 1 μ Ci of [γ - 32 P] ATP) followed by incubation for 20 min at 30°C. Reactions were stopped by the addition of 50% acetic acid, spotted to 2 x 2 cm² of P81 cellulose paper, washed with 50 mM phosphoric acid (H₃PO₄), and then air-dried. 32 P incorporation was quantitated by liquid scintillation counting. All assays were repeated on three independent occasions and statistical differences in inhibition were determined by Student's paired t-test.

2.7. Nuclear Magnetic Resonance – Saturation Transfer Difference

Competitive Saturation Transfer Difference (STD) NMR experiments were performed on a Bruker Avance II 800 MHz NMR spectrometer equipped with a z-gradient 5 mm triple resonance cryoprobe. Samples of 300 μ M AMP-PNP (adenosine 5'-(β , γ -imido)triphosphate) and TIJIP were prepared with 10 μ M JNK1 in a 50 mM phosphate buffer supplemented with 20 mM MgCl₂ and 5 mM DTT, pH 6.8. To minimize volume changes in the competition experiment, a 1.5 μ l aliquot of 100 mM JD123 stock solution in DMSO was added to the 500 μ l NMR sample to give final concentration of 300 μ M JD123. The STD NMR spectra were acquired at 25 °C using a standard 1D STD pulse sequence [44]: a 3 s saturation pulse using a train of 50 ms Gaussian pulses with a B₁ field of about 130 Hz, separated by 1 ms delays. An additional 2 s relaxation delay was used. The on- and off-resonance frequencies were -1.5 and

-25 ppm, respectively. To suppress residual protein signals, a spin-lock pulse of 20 ms was employed, and water suppression was by a 3-9-19 pulse sequence.

2.8. Cell Culture, treatment, lysis and immunoblotting

Murine embryonic fibroblasts (MEFs) were exposed to hyperosmotic stress (0.5 M sorbitol, 60 min) and lysates prepared as described previously [45]. Where indicated, cells were pre-treated with either vehicle (DMSO), JD123 (50 and 100 μ M) or JD124 (50 and 100 μ M) for 30 min prior to hyperosmotic stress. Cell lysates were prepared in RIPA buffer (50 mM Tris-HCl, pH 7.3, 150 mM NaCl, 0.1 mM EDTA, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 0.2% (w/v) NaF and 100 μ M Na_3VO_4) supplemented with protease inhibitors. After 10 min on ice, cell debris was removed by centrifugation (14 000 x g, 10 min). Protein concentrations were then determined by Bradford assay. Protein lysates (30 μ g) were resolved by SDS-PAGE, transferred onto polyvinylidene fluoride (PVDF) membranes and immunoblotted with antibodies recognizing phosphorylated cJun (p-cJun(Ser63), cJun, phosphorylated JNK (p-JNK) (Cell Signaling) and JNK (BD Biosciences Australia). Experiments were repeated on three independent occasions with comparable results, and data quantitation was conducted using ImageJ software.

3. Results

3.1 Virtual Screening Directed to Identify RP site binders - the JD100 Series

Our previous work identified four residues in TIJIP critical for JNK inhibition, these corresponding to ¹⁵⁶Arg, ¹⁵⁷Pro, ¹⁶⁰Leu and ¹⁶²Leu within the full length JIP1 sequence [31, 33]. Using the available structure of JNK1α1 bound to the JIP1-derived JNK inhibitory peptide (Protein Data Bank (PDB): 1ukh) [32], we further divided this docking site of JNK1 into two regions and focused our docking studies towards the region of JNK1 interacting with ¹⁵⁶Arg-¹⁵⁷Pro (referred throughout as the “RP site”) (Figure 1A). This JNK1 “RP site” is characterized by a shallow hydrophobic pocket and a series of acidic (³²⁹Glu, ³²⁶Asp) and polar (¹³⁰Tyr, ¹³³Tyr) residues that interact with the ¹⁵⁶Arg side-chain within the JIP1 peptide [32] (Figure 1B). Our initial virtual screen of the ZINC chemical databases yielded a set of several different chemotypes predicted as binders for the ¹⁵⁶Arg-¹⁵⁷Pro binding region of JNK1. Our priority for testing was directed to chemotypes that possessed a small ring system and either charged or hydrogen bond-donating substituents.

In our first round of testing, inhibitors of JNK1 activity towards cJun were identified as chemotypes typified by an aryl-substituted aminothiazole series of compounds (Supplementary Table 1, see JD100-116 for the initial series tested, and Supplementary Figure 1 for an overlay of active hits 105, 109, 110). Compounds 109 and 110 share a common binding mode with 105, being more rigid and occupying the binding site in a different manner. In the protein binding site microenvironment these aryl-substituted aminothiazoles might also be at least partially protonated with the protonated aminothiazole moiety predicted to bind in the same negatively charged region normally occupied by ¹⁵⁶Arg

side-chain within TIJIP. The neutral species would also be able to make the corresponding hydrogen bonding contacts so accordingly both neutral and protonated species were considered during the docking. A subsequent search of the Chembridge database for analogues containing aminothiazole groups identified additional commercially available compounds, 12 of which were selected for the second round of testing (Supplementary Table 1, see JD117-128), which also satisfied our solubility and logP criteria. The results from the testing of 7 of these compounds for their actions to inhibit JNK1 activity towards cJun are shown in Figure 2A, when tested on three independent occasions. The structure of each of these compounds is shown in Figure 2B. Two compounds (JD123 and JD118) showed the most effective inhibition of JNK under these assay conditions, two compounds (JD122 and JD127) showed intermediate effects, and lastly three compounds (JD117, JD119 and JD124) showed no statistically significant effects on JNK1 activity under the conditions of assay (Figure 2A). The levels of inhibition noted for 100 μ M JD123 or JD118 were >80% and thus comparable to the level of inhibition achieved by 2 μ M TIJIP peptide when assayed under these conditions (Figure 2A). This prompted our further evaluation of the mechanism of action and specificity of JD123 as a JNK inhibitor, and the more general kinase inhibitory properties of the bi-thiazole-2,2'-amine structure.

3.2. Mechanism of JD123 inhibition towards JNK1: molecular modeling, biochemical and structural analysis show JD123 targeting JNK1 "RP (substrate) site" and ATP-binding site

JD123, (4'-methyl-N²-3-pyridinyl-4,5'-bi-1,3-thiazole-2,2'-diamine; Chembridge ID: 7850865), was chosen for further characterization of its actions as a JNK inhibitory compound. We first assessed its likely modes of interaction with JNK1 via molecular docking protocols. The JNK1 protein surface was generated using GOLD (v.3.2) and all acidic (red)

and basic (blue) residues identified. The possible protonation states for JD123 that may be recognized in the protein microenvironment are presented in Figure 3A. pKa simulations of JD123 (MarvinSketch™) suggest low populations of protonated species distributed over the aminothiazole ring nitrogen and pyridine nitrogen. Only the possible protonation state of the aminothiazole is considered here as it mimics the interaction of the JIP arginine side-chain. The docking poses of the neutral and protonated forms of JD123 in the JNK1 “RP site” and ATP-site reveal several possible docking conformations (Figure 3Bi upper and lower panels). Firstly, the neutral form of the inhibitor was observed however to bind in 3 different configurations due to the lack of the extra hydrogen bond donor seen in the protonated form (Figure 3Bi, top panel). As a result the neutral molecule binds either with the hydrophobic section occupying the narrow groove adjacent to but not occupied by JIP, and a hydrogen bond of the amino group to the ³²⁴Trp carbonyl (cluster 1), or two different almost mirror image conformations in the shallow pocket with the amino group bridging between Glu329 and the ³²⁴Trp carbonyl (clusters 2 and 3) (Figure 3Bi). Meanwhile, the protonated form bound in a single conformation, intimately interacting with the acidic arginine-binding pocket composed of the side chain carboxylates of ³²⁶Asp and ³²⁹Glu, as well as the hydroxyl of ¹³⁰Tyr and the main chain carbonyl of ³²⁴Trp (Figure 3Bi, bottom panel). The pyridine ring occupied the shallow pocket that normally binds the proline ring of JIP (Figure 3Bi). Furthermore, when examining the possible binding of JD123 to the JNK ATP-binding site, the calculated docking scores were similar to those generated for docking of JD123 to the “RP site” with the generated model (Figure 3Ci), indicating that JD123 could fit in the ATP-binding pocket of JNK1. Taken together, these docking studies revealed the importance of considering all possible docking modes and interaction sites for a small molecule inhibitor and prompted further biochemical assessment of the possible modes of JNK1 inhibition by JD123.

To evaluate a possible dual-inhibitory mechanism of JD123 towards JNK1, we performed kinetic analysis under varying substrate conditions. We analyzed JNK1 α 1 activity with either GST-cJun (1–135) or ATP concentrations varied at a fixed concentration of the other substrate in the presence or absence of a range of concentrations of JD123 (0, 10 - 40 μ M). Data obtained from experiments performed at fixed ATP concentration, where GST-cJun (1–135) concentrations were varied in the presence of increasing concentrations of JD123, indicative of mixed-competitive inhibition (Figure 3Bii). When similar experiments were performed with a constant GST-cJun (1–135) concentration, varied ATP concentrations, and with several concentrations of JD123, analysis confirmed the inhibition was competitive with respect to ATP (Figure 3Cii). These kinetics data were similar to those for other JNK inhibitors reported to be dual-inhibitory in their actions [43, 46].

To confirm the actions of JD123 to compete with both peptide and ATP substrate analogues, we conducted competition Saturation Transfer Difference (STD) NMR experiments that would directly report the in-solution interactions with JNK1 independently of the measurement of effects on kinase activities. Specifically, we evaluated interactions of AMP-PNP (reporting on the ATP-binding site) and TIJIP (reporting on the protein substrate binding site) with JNK1, and the impact of the presence of JD123. Thus, a reference NMR spectrum of AMP-PNP, a non-hydrolysable analogue of ATP was recorded (Figure 4A), followed by a Saturation Transfer Difference (STD) NMR spectrum in the presence of 10 μ M JNK1 (Figure 4B). The observed STD enhancement was consistent with AMP-PNP binding weakly, as expected from the reported K_D of $\sim$ 20 μ M for ATP [32]. Upon the addition of JD123, a significant STD enhancement was observed for JD123, whereas no STD effect was observed for AMP-PNP, consistent with JD123 competing with and binding more tightly than AMP-

PNP (Figure 4C). Similarly, a reference spectrum for TIJIP (Figure 4D) was followed by an STD NMR spectrum of TIJIP in the presence of 10 μ M JNK1 (Figure 4E). STD enhancements for the methyl signals of the two Leu residues, the methyls of the two Thr residues, and the aromatic ring of the Phe residue were 1.6%, 1.11% and 1.6%, respectively (Figure 4E). On the addition of JD123, these enhancements were reduced to 1.2%, 0.8% and 1.1%, respectively (Figure 4F). This reduction in the STD enhancement was consistent with JD123 competing with TIJIP, and was consistent with the measured affinity (0.4 μ M) of TIJIP for this site [47]. Taken together, these studies confirm the direct interaction of JD123 with the ATP-binding pocket as well as substrate binding site of JNK1.

3.3. *In vitro* characterization of JD123 as a JNK inhibitory compound.

We next evaluated the efficacy of JD123 to inhibit JNK1-mediated phosphorylation of three well characterized transcription factor substrates, cJun (Figure 5A), ATF2 (Figure 5B), Elk1 (Figure 5C). In agreement with our previous results showing TIJIP as a JNK inhibitory peptide, JD123 inhibited JNK activity towards all three substrates. Whilst statistically significant inhibition was observed for most reactions that included 10 μ M JD123, the most potent inhibition was observed when JD123 was included at a final concentration of 100 μ M in these reactions (Figure 5A-C).

To extend these studies, we considered whether JD123 inhibited JNK activity towards other substrates. A survey of the available literature describing JNK substrates [11], suggested that the microtubule-regulatory protein DCX represents a distinct class of substrate due to its interaction via its doublecortin (DC) domains with JNK [48]. When we tested JNK1-mediated phosphorylation of DCX, we observed significant inhibition by JD123 and the TIJIP peptide (Figure 5D). Kinetic analyses showed ATP-competitive inhibition of JD123 towards JNK1-

mediated DCX phosphorylation (Supplementary Figure 2). Similar efficacy of JD123-mediated inhibition was also observed for the related JNK2 and JNK3 towards the cJun, ATF2, Elk1 and DCX substrate proteins (Supplementary Figures 3 and 4). Thus, JD123 inhibits the activity of all three JNK isoforms towards a range of protein substrates. Furthermore, when we tested the efficacy of JD123 as a JNK inhibitor in cells, we noted that concentrations of JD123 and JD124 up to 100 μ M showed no cell toxicity (Ngoei and Bogoyevitch, unpublished). Immunoblotting revealed that the compounds did not interfere with the phosphorylation of JNK, and that JD123, but not JD124, inhibited c-Jun Ser63 phosphorylation in this murine embryonic fibroblast cell system in a dose-dependent manner (Figure 6A, B), consistent with the inhibition of JNK activities toward c-Jun.

To assess the mode of JNK inhibition by JD123 further, we assayed the impact of JD123 on JNK1 activity towards a peptide substrate based on the Epidermal Growth Factor Receptor (EGFR). This peptide substrate has no reported consensus JBD motif critical for interaction towards JNK [11, 43] and thus recently has been effectively used in the *in vitro* testing of JNK ATP-competitive inhibitors [43]. We observed that JD123 inhibited JNK phosphorylation of the EGFR peptide in a concentration-dependent manner (Figure 7). Moreover, the commercially available ATP-competitive JNK inhibitor VIII provided significant inhibition at 10 μ M under these assay conditions (Figure 7). As shown previously [43], the peptide inhibitor TIJIP did not inhibit JNK phosphorylation towards EGFR peptide, consistent with its substrate docking site-competitive nature for JNK inhibition (Figure 7).

3.4. *In vitro* assays reveal JD123 inhibition of other protein kinases

With a characterized ATP-competitive mode of JNK1 inhibition by JD123, we assessed its actions on the related MAPKs ERK1 and ERK2. Under our assay conditions, ERK2 showed lower activity than ERK1 towards the Elk protein substrate, but JD123 (10 - 100 μ M) did not inhibit ERK1 or ERK2 activity (Figure 8A, B). TIJIP, included as a negative control, did not inhibit ERK1/2 activity towards Elk1 (Figure 8A, B).

To extend these studies, JD123 and JD124 were tested against a panel of protein kinases (National Centre for Protein Kinase Profiling, MRC Protein Phosphorylation Unit, University of Dundee). Whilst JNK1, JNK2 and JNK3 were inhibited by up to 50% under these assay conditions including 100 μ M JD123, 100 μ M JD124 showed comparatively less inhibition (Table 1), thus confirming the specificity noted in our preceding analysis (Figure 2A). Only minimal inhibitory effects were noted for JD123 (10 or 100 μ M) on the activities of the widely studied p38- α and p38- β isoforms (Table 1). Under these assay conditions, the inclusion of the p38 inhibitor SB203580 provided robust inhibition in quality control assays run together with the test assays (National Centre for Protein Kinase Profiling, results not shown). Of the lesser-studied p38 isoforms, p38- δ showed >50% inhibition by 100 μ M JD123 whereas p38- γ was inhibited by 85% by 10 μ M JD123 (Table 1). JD124 (100 μ M) only inhibited p38- γ activity towards MBP substrate by 40% under these conditions (Table 1). The assays for ERK1 and ERK2 revealed JD123 and JD124 mediated inhibition of ERK1, but not ERK2, towards the MBP substrate protein.

Thus, to verify the effects of JD123 to inhibit p38- γ , we conducted *in vitro* activity assays using the ATF2 protein as a substrate. We demonstrated that JD123 effectively inhibited p38- γ activity in a dose-dependent manner (Figure 8Ci). A JNK-specific peptide inhibitor, TIJIP, included in the assay as a negative control showed no inhibition of p38- γ (Figure 8Ci).

Kinetic analyses with varying ATP concentrations varied at a fixed concentration of GST-ATF2 in the presence of increasing concentrations of JD123 (0-10 μ M) showed ATP-competitive inhibition of p38- γ (Figure 8Cii).

Additional results of the kinase inhibitory screen to evaluate the broader extent of kinase inhibition by 10 μ M JD123 are further presented in Table 2. The full list of the abbreviations for the kinases tested is presented in Supplementary Table 2. Under these standard conditions of assay, seven of the 100 additional kinases tested were inhibited by >70% in the presence of 10 μ M JD123, namely PKB β /Akt β , PIM3, CLK2, CAMK1, PIM1, MAPKAPK3 and BTK (Table 2). Conversely, 55 kinases were inhibited by < 30% under these assay conditions (Table 2). Additional assays were also conducted with the inclusion of the higher concentration of JD123 (100 μ M) as well as testing whether JD124 (100 μ M) was also inhibitory towards these kinases. As shown in Supplementary Table 3 for the top 25 kinases inhibited by 10 μ M JD123, there was a general trend that greater inhibition was observed with 100 μ M JD123, however in most cases there was comparable inhibition also observed with 100 μ M JD124 and thus there was not the discrimination of effects noted for JD123 and JD124 for JNK or p38- γ inhibition (Table 1). Exceptions were IR1 and PLK1 that were inhibited by 100 μ M JD123, but that showed no significant inhibition in the presence of 100 μ M JD124 (Supplementary Table 3). The greater inhibition by 100 μ M inhibitors further prompted the definition of the kinases inhibited by $\geq$ 90% by 100 μ M JD123 and/or JD124, and this analysis revealed 20 kinases (Supplementary Table 4).

4. Discussion

Protein kinases, with their critical roles in intracellular signaling pathways controlling the range of cellular activities, are an important group of therapeutic targets. High-throughput *in vitro* screens of large libraries of small, and often drug-like, molecules have been routinely employed in the discovery of protein kinase inhibitors [25, 49, 50]. Here we have taken the strategy of structure-based virtual screening of available small molecule libraries, and identified thiazole-2,2'-diamines as a new class of protein kinase inhibitor scaffold.

Structure-based virtual screening approaches have previously identified inhibitors for other protein kinases including death-associated protein kinases (DAPK1) [51], Aurora-B Kinase [52] protein kinase B/Akt [53], p38 MAPKs [54], the EGFR [55] and Casein Kinase 2 [56]. Despite the recent successes of comprehensive kinase inhibitor screens [36, 57] that have revealed critical features of kinase inhibitor selectivity, the popularity and application of virtual screening to discover new classes of inhibitors of protein kinases is likely to increase, particularly with the continued improvements in docking algorithms/programs, refined small molecule libraries, improved access to higher computing power and a larger range of solved protein kinase structures [58, 59]. Importantly, consideration of molecular dynamics that improve our understanding of the conformational heterogeneity of regions such as the activation loop of the kinase will further improve success [60].

Our evaluation of the properties of a lead compound identified in our screen for JNK-docking small molecules, herein referred to as JD123, revealed an unexpected ATP-competitive inhibition of JNK1. Our analyses highlight the critical importance for biochemical characterization, including kinetic analysis of inhibition under varying ATP and protein substrate conditions. The observation that JD123 did not show simple competitive inhibition at the substrate site is likely related to the protonation state of the aminothiazole unit. Whilst a

fully protonated species may be more potent at this site it is generally considered that fully charged arginine mimetics are less druglike entities. Notably, a related structural analog, JD124, did not show significant inhibition of JNK *in vitro*, consistent with docking analysis that the methyl group of the JD124 pyridine ring created some steric hindrance towards the shallow pocket of JNK substrate and more steric hindrance towards the ATP binding sites (Supplementary Figure 5). Further chemical modification of JD123 will be critical for more precisely defining the structure-activity relationships with respect to the ATP binding and protein substrate binding sites of JNK.

ATP competitive inhibitors of JNKs have been exploited in the evaluation of the biological significance of JNK signaling (see review [25, 49, 50]). Notably, the anthrapyrazole compound SP600125 [26], was the first commercially available ATP-competitive JNK inhibitor. Despite its recognized off-target effects identified in protein kinase activity profiling where it inhibited 13 of 30 kinases tested with 6 inhibited more potently than JNK [42], and interactions with 39 of 113 kinases evaluated in a phage based kinase interaction study [61], SP600125 continues to be widely used as a JNK-selective inhibitor. In evaluating our inhibition data for JD123, the parallel quality control data for SP600125 towards the panel of kinases revealed the striking ability of SP600125 to act as a broad kinase inhibitor when assayed at dose of 1 μ M which affords ~50% inhibition of JNK1, 2 and 3 under these assay conditions, or the higher dose of 10 μ M that provides more robust JNK inhibition (Supplementary Table 5).

A number of dual inhibitors of JNK are now characterised [43, 46]. These molecules are predicted to bind to both substrate and ATP-binding sites, however more detailed structural studies that include co-crystallisation of JNK with these inhibitors will confirm the details of

their interactions with both sites. Similarly, structural analysis of the JD123-JNK1 complex will be necessary to confirm the precise docking modes and interactions with JNK1. A number of thiazole-based small molecule JNK inhibitors have also been reported from high-throughput screens [62, 63]. The first thiazole compound known as BI87G3 (2-(5-nitrothiazol-2-ylthio)benzo[d]thiazole) displayed substrate competition with its nitrothiazole moiety facing towards ^{127}Arg and ^{163}Cys of JNK1 in addition to hydrogen bonding between benzothiazole and ^{127}Arg which allows access into the adjacent sub-pocket [62]. BI87G3 can inhibit $\text{TNF}\alpha$ -stimulated cJun phosphorylation in HeLa cells [62]. Through extensive structure-activity relationship studies, Elan Pharmaceuticals has discovered another ATP-competitive, thiazole-based compound that showed 10-fold selectivity on JNK1 and JNK3 over JNK2 [63]. Our detailed analysis of the interaction of JNK1 with JD123 using STD NMR highlighted the binding of JD123 with both the ATP-binding site and the protein substrate site, and confirmed the power of this independent approach to address ligand interactions with the JNK1 protein.

A further important consideration in protein kinase inhibitor development is selectivity within the kinome space. As noted, the conservation of the ATP-binding site of kinases may compromise, but does not preclude, the attainment of highly specific ATP-competitive inhibitors of protein kinases [36, 57]. Our results here with JD123 show inhibition of both JNK1, JNK2 and JNK3. A broader screen against 100 protein kinases showed the actions of JD123 to inhibit ERK1, but not ERK2, however this inhibition of ERK1 was not reproduced in our assays using the physiological substrate, Elk1. Furthermore, JD123 was shown to inhibit the p38- γ whereas p38- α , β and δ isoforms were unaffected. Our kinetics analysis confirmed JD123 to be an ATP-competitive inhibitor of p38- γ . The ability to probe the actions of p38- α/β has been greatly facilitated by the availability of the pyridinyl-imidazole

ATP-competitive inhibitor, SB203580 [64, 65] and related compounds [66] as reviewed [67]. The importance of modulating p38- γ actions has been recently shown with the implication of p38- γ in cancer cell motility and metastasis [68], the loss of sensitivity to poly(ADP-ribose) polymerase 1 inhibition [69], and changes in mitosis and cell death [70]. Thus, inhibition of p38- γ by JD123 suggests a new lead, and therefore a new route to anticancer therapeutics.

However, JD123-mediated inhibition of several other kinases was also noted in this broader screening strategy. Notable amongst these kinases were the Aurora kinases A and B as well as PKB β /Akt β . More detailed dose response evaluations of the inhibition of these kinases is clearly warranted. Of further interest has been the disclosure by Novartis and Merck Serono (WO 2009/080705 and WO 2005/068444, respectively) of bi-thiazoles as inhibitors of phosphatidyl inositol 3-kinase (PI3K). Whilst these additional targets will likely complicate the interpretation of cell-based assays of JD123 effects, these results suggest the use of thiazole diamines as a new scaffold for the development of new classes of small molecule ATP-competitive kinase inhibitors with the possibility that its broader range of kinase targets may enhance its efficacy to prevent cell proliferation, movement and survival.

Taken together, our results highlight the strengths of structure-based virtual screening approaches to identify kinase inhibitors. Detailed evaluation of the modes of interaction, combined with biochemical analysis, has revealed a thiazole-2,2' diamine, as an ATP-competitive of JNK1, with further more extensive screening against a broader range of kinases indicating the possibility that appropriately modified thiazole diamines will provide a new chemical scaffold for other protein kinases. The challenge now lies in exploiting the ATP-binding site and protein substrate-binding site pharmacophores to develop new classes of inhibitors with increased potency towards selected kinases.

Acknowledgements:

This work was supported by an ARC Discovery grant (DP0984225 to MAB). KRWN acknowledges the support of a Melbourne International Research Scholarship (MIRS), a Melbourne International Fee Remission Scholarship (MIRS) and a Melbourne Research Scholarship (MRS). DN is supported by the University of Melbourne CR Roper Bequest Fellowship.

References

- [1] J.B. Stock, A.J. Ninfa, A.M. Stock, Protein phosphorylation and regulation of adaptive responses in bacteria, *Microbiol Rev*, 53 (1989) 450-490.
- [2] C. Chang, R.C. Stewart, The two-component system. Regulation of diverse signaling pathways in prokaryotes and eukaryotes, *Plant Physiol*, 117 (1998) 723-731.
- [3] D. Ribet, P. Cossart, Post-translational modifications in host cells during bacterial infection, *FEBS Lett*, 584 (2010) 2748-2758.
- [4] S. Caenepeel, G. Charyczak, S. Sudarsanam, T. Hunter, G. Manning, The mouse kinome: discovery and comparative genomics of all mouse protein kinases, *Proc Natl Acad Sci U S A*, 101 (2004) 11707-11712.
- [5] J.M. Goldberg, G. Manning, A. Liu, P. Fey, K.E. Pilcher, Y. Xu, J.L. Smith, The dictyostelium kinome--analysis of the protein kinases from a simple model organism, *PLoS Genet*, 2 (2006) e38.
- [6] G. Manning, D.B. Whyte, R. Martinez, T. Hunter, S. Sudarsanam, The protein kinase complement of the human genome, *Science*, 298 (2002) 1912-1934.
- [7] G. Manning, G.D. Plowman, T. Hunter, S. Sudarsanam, Evolution of protein kinase signaling from yeast to man, *Trends Biochem Sci*, 27 (2002) 514-520.
- [8] M.A. Bogoyevitch, N.W. Court, Counting on mitogen-activated protein kinases--ERKs 3, 4, 5, 6, 7 and 8, *Cell Signal*, 16 (2004) 1345-1354.
- [9] J.M. Kyriakis, P. Banerjee, E. Nikolakaki, T. Dai, E.A. Rubie, M.F. Ahmad, J. Avruch, J.R. Woodgett, The stress-activated protein kinase subfamily of c-Jun kinases, *Nature*, 369 (1994) 156-160.
- [10] T. Kallunki, B. Su, I. Tsigelny, H.K. Sluss, B. Derijard, G. Moore, R. Davis, M. Karin, JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation, *Genes Dev*, 8 (1994) 2996-3007.

- [11] M.A. Bogoyevitch, B. Kobe, Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases, *Microbiol Mol Biol Rev*, 70 (2006) 1061-1095.
- [12] S. Gupta, T. Barrett, A.J. Whitmarsh, J. Cavanagh, H.K. Sluss, B. Derijard, R.J. Davis, Selective interaction of JNK protein kinase isoforms with transcription factors, *EMBO J*, 15 (1996) 2760-2770.
- [13] T. Borsello, P.G. Clarke, L. Hirt, A. Vercelli, M. Repici, D.F. Schorderet, J. Bogousslavsky, C. Bonny, A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia, *Nat Med*, 9 (2003) 1180-1186.
- [14] D.D. Yang, C.Y. Kuan, A.J. Whitmarsh, M. Rincon, T.S. Zheng, R.J. Davis, P. Rakic, R.A. Flavell, Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the *Jnk3* gene, *Nature*, 389 (1997) 865-870.
- [15] X. Zhu, A.K. Raina, C.A. Rottkamp, G. Aliev, G. Perry, H. Boux, M.A. Smith, Activation and redistribution of c-jun N-terminal kinase/stress activated protein kinase in degenerating neurons in Alzheimer's disease, *J Neurochem*, 76 (2001) 435-441.
- [16] J.J. Pei, E. Braak, H. Braak, I. Grundke-Iqbal, K. Iqbal, B. Winblad, R.F. Cowburn, Localization of active forms of C-jun kinase (JNK) and p38 kinase in Alzheimer's disease brains at different stages of neurofibrillary degeneration, *J Alzheimers Dis*, 3 (2001) 41-48.
- [17] S. Hunot, M. Vila, P. Teismann, R.J. Davis, E.C. Hirsch, S. Przedborski, P. Rakic, R.A. Flavell, JNK-mediated induction of cyclooxygenase 2 is required for neurodegeneration in a mouse model of Parkinson's disease, *Proc Natl Acad Sci U S A*, 101 (2004) 665-670.
- [18] J. Hirosumi, G. Tuncman, L. Chang, C.Z. Gorgun, K.T. Uysal, K. Maeda, M. Karin, G.S. Hotamisligil, A central role for JNK in obesity and insulin resistance, *Nature*, 420 (2002) 333-336.

- [19] X.X. Yu, S.F. Murray, L. Watts, S.L. Booten, J. Tokorcheck, B.P. Monia, S. Bhanot, Reduction of JNK1 expression with antisense oligonucleotide improves adiposity in obese mice, *Am J Physiol Endocrinol Metab*, 295 (2008) E436-445.
- [20] J.L. Swantek, M.H. Cobb, T.D. Geppert, Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for lipopolysaccharide stimulation of tumor necrosis factor alpha (TNF-alpha) translation: glucocorticoids inhibit TNF-alpha translation by blocking JNK/SAPK, *Mol Cell Biol*, 17 (1997) 6274-6282.
- [21] Z. Han, D.L. Boyle, L. Chang, B. Bennett, M. Karin, L. Yang, A.M. Manning, G.S. Firestein, c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis, *J Clin Invest*, 108 (2001) 73-81.
- [22] P.K. Roy, F. Rashid, J. Bragg, J.A. Ibdah, Role of the JNK signal transduction pathway in inflammatory bowel disease, *World J Gastroenterol*, 14 (2008) 200-202.
- [23] N.J. Kennedy, R.J. Davis, Role of JNK in tumor development, *Cell Cycle*, 2 (2003) 199-201.
- [24] A.S. Nateri, B. Spencer-Dene, A. Behrens, Interaction of phosphorylated c-Jun with TCF4 regulates intestinal cancer development, *Nature*, 437 (2005) 281-285.
- [25] M.A. Bogoyevitch, P.G. Arthur, Inhibitors of c-Jun N-terminal kinases: JunK no more?, *Biochim Biophys Acta*, 1784 (2008) 76-93.
- [26] B.L. Bennett, D.T. Sasaki, B.W. Murray, E.C. O'Leary, S.T. Sakata, W. Xu, J.C. Leisten, A. Motiwala, S. Pierce, Y. Satoh, S.S. Bhagwat, A.M. Manning, D.W. Anderson, SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase, *Proc Natl Acad Sci U S A*, 98 (2001) 13681-13686.
- [27] S. Carboni, A. Hiver, C. Szyndralewicz, P. Gaillard, J.P. Gotteland, P.A. Vitte, AS601245 (1,3-benzothiazol-2-yl (2-[[2-(3-pyridinyl) ethyl] amino]-4 pyrimidinyl)

acetonitrile): a c-Jun NH₂-terminal protein kinase inhibitor with neuroprotective properties, *J Pharmacol Exp Ther*, 310 (2004) 25-32.

[28] B.G. Szczepankiewicz, C. Kosogof, L.T. Nelson, G. Liu, B. Liu, H. Zhao, M.D. Serby, Z. Xin, M. Liu, R.J. Gum, D.L. Haasch, S. Wang, J.E. Clampit, E.F. Johnson, T.H. Lubben, M.A. Stashko, E.T. Olejniczak, C. Sun, S.A. Dorwin, K. Haskins, C. Abad-Zapatero, E.H. Fry, C.W. Hutchins, H.L. Sham, C.M. Rondinone, J.M. Trevillyan, Aminopyridine-based c-Jun N-terminal kinase inhibitors with cellular activity and minimal cross-kinase activity, *J Med Chem*, 49 (2006) 3563-3580.

[29] V.P. Krenitsky, M. Delgado, L. Nadolny, K. Sahasrabudhe, L. Ayala, S.S. Clareen, R. Hilgraf, R. Albers, A. Kois, K. Hughes, J. Wright, J. Nowakowski, E. Sudbeck, S. Ghosh, S. Bahmanyar, P. Chamberlain, J. Muir, B.E. Cathers, D. Giegel, L. Xu, M. Celeridad, M. Moghaddam, O. Khatsenko, P. Omholt, J. Katz, S. Pai, R. Fan, Y. Tang, M.A. Shirley, B. Benish, K. Blease, H. Raymon, S. Bhagwat, I. Henderson, A.G. Cole, B. Bennett, Y. Satoh, Aminopurine based JNK inhibitors for the prevention of ischemia reperfusion injury, *Bioorg Med Chem Lett*, 22 (2012) 1427-1432.

[30] M.A. Bogoyevitch, K.R. Ngoei, T.T. Zhao, Y.Y. Yeap, D.C. Ng, c-Jun N-terminal kinase (JNK) signaling: recent advances and challenges, *Biochim Biophys Acta*, 1804 (2010) 463-475.

[31] R.K. Barr, T.S. Kendrick, M.A. Bogoyevitch, Identification of the critical features of a small peptide inhibitor of JNK activity, *J Biol Chem*, 277 (2002) 10987-10997.

[32] Y.S. Heo, S.K. Kim, C.I. Seo, Y.K. Kim, B.J. Sung, H.S. Lee, J.I. Lee, S.Y. Park, J.H. Kim, K.Y. Hwang, Y.L. Hyun, Y.H. Jeon, S. Ro, J.M. Cho, T.G. Lee, C.H. Yang, Structural basis for the selective inhibition of JNK1 by the scaffolding protein JIP1 and SP600125, *EMBO J*, 23 (2004) 2185-2195.

- [33] R.K. Barr, I. Boehm, P.V. Attwood, P.M. Watt, M.A. Bogoyevitch, The critical features and the mechanism of inhibition of a kinase interaction motif-based peptide inhibitor of JNK, *J Biol Chem*, 279 (2004) 36327-36338.
- [34] H. Kaneto, Y. Nakatani, T. Miyatsuka, D. Kawamori, T.A. Matsuoka, M. Matsuhisa, Y. Kajimoto, H. Ichijo, Y. Yamasaki, M. Hori, Possible novel therapy for diabetes with cell-permeable JNK-inhibitory peptide, *Nat Med*, 10 (2004) 1128-1132.
- [35] K. Reinecke, S. Eminel, F. Dierck, W. Roessner, S. Kersting, A.M. Chromik, O. Gavrilova, A. Laukeviciene, I. Leuschner, V. Waetzig, P. Rosenstiel, T. Herdegen, C. Sina, The JNK inhibitor XG-102 protects against TNBS-induced colitis, *PLoS One*, 7 (2012) e30985.
- [36] T. Anastassiadis, S.W. Deacon, K. Devarajan, H. Ma, J.R. Peterson, Comprehensive assay of kinase catalytic activity reveals features of kinase inhibitor selectivity, *Nat Biotechnol*, 29 (2011) 1039-1045.
- [37] C. Zhang, G. Habets, G. Bollag, Interrogating the kinome, *Nat Biotechnol*, 29 (2011) 981-983.
- [38] J.L. Stebbins, S.K. De, T. Machleidt, B. Becattini, J. Vazquez, C. Kuntzen, L.H. Chen, J.F. Cellitti, M. Riel-Mehan, A. Emdadi, G. Solinas, M. Karin, M. Pellecchia, Identification of a new JNK inhibitor targeting the JNK-JIP interaction site, *Proc Natl Acad Sci U S A*, 105 (2008) 16809-16813.
- [39] R. Akella, T.M. Moon, E.J. Goldsmith, Unique MAP Kinase binding sites, *Biochim Biophys Acta*, 1784 (2008) 48-55.
- [40] M.L. Verdonk, J.C. Cole, M.J. Hartshorn, C.W. Murray, R.D. Taylor, Improved protein-ligand docking using GOLD, *Proteins*, 52 (2003) 609-623.

- [41] K.R. Ngoei, B. Catimel, N. Church, D.S. Lio, C. Dogovski, M.A. Perugini, P.M. Watt, H.C. Cheng, D.C. Ng, M.A. Bogoyevitch, Characterization of a novel JNK (c-Jun N-terminal kinase) inhibitory peptide, *Biochem J*, 434 (2011) 399-413.
- [42] J. Bain, L. Plater, M. Elliott, N. Shpiro, C.J. Hastie, H. McLauchlan, I. Klevernic, J.S. Arthur, D.R. Alessi, P. Cohen, The selectivity of protein kinase inhibitors: a further update, *Biochem J*, 408 (2007) 297-315.
- [43] T. Chen, N. Kablaoui, J. Little, S. Timofeevski, W.R. Tschantz, P. Chen, J. Feng, M. Charlton, R. Stanton, P. Bauer, Identification of small-molecule inhibitors of the JIP-JNK interaction, *Biochem J*, 420 (2009) 283-294.
- [44] M. Mayer, B. Meyer, Characterization of Ligand Binding by Saturation Transfer Difference NMR Spectroscopy, *Angew Chem Int Ed* 38 (1999) 1784-1788.
- [45] D.C. Ng, T.T. Zhao, Y.Y. Yeap, K.R. Ngoei, M.A. Bogoyevitch, c-Jun N-terminal kinase phosphorylation of stathmin confers protection against cellular stress, *J Biol Chem*, 285 (2010) 29001-29013.
- [46] S.K. De, E. Barile, V. Chen, J.L. Stebbins, J.F. Cellitti, T. Machleidt, C.B. Carlson, L. Yang, R. Dahl, M. Pellecchia, Design, synthesis, and structure-activity relationship studies of thiophene-3-carboxamide derivatives as dual inhibitors of the c-Jun N-terminal kinase, *Bioorg Med Chem*, 19 (2011) 2582-2588.
- [47] C. Yan, T. Kaoud, S. Lee, K.N. Dalby, P. Ren, Understanding the specificity of a docking interaction between JNK1 and the scaffolding protein JIP1, *J Phys Chem B*, 115 (2011) 1491-1502.
- [48] A. Gdalyahu, I. Ghosh, T. Levy, T. Sapir, S. Sapoznik, Y. Fishler, D. Azoulai, O. Reiner, DCX, a new mediator of the JNK pathway, *EMBO J*, 23 (2004) 823-832.
- [49] K. Burkhard, S. Smith, R. Deshmukh, A.D. MacKerell, Jr., P. Shapiro, Development of extracellular signal-regulated kinase inhibitors, *Curr Top Med Chem*, 9 (2009) 678-689.

- [50] R.M. Eglén, T. Reisine, The current status of drug discovery against the human kinome, *Assay Drug Dev Technol*, 7 (2009) 22-43.
- [51] M. Okamoto, K. Takayama, T. Shimizu, K. Ishida, O. Takahashi, T. Furuya, Identification of death-associated protein kinases inhibitors using structure-based virtual screening, *J Med Chem*, 52 (2009) 7323-7327.
- [52] D.H. Fu, W. Jiang, J.T. Zheng, G.Y. Zhao, Y. Li, H. Yi, Z.R. Li, J.D. Jiang, K.Q. Yang, Y. Wang, S.Y. Si, Jadomycin B, an Aurora-B kinase inhibitor discovered through virtual screening, *Mol Cancer Ther*, 7 (2008) 2386-2393.
- [53] J.L. Medina-Franco, M.A. Giulianotti, Y. Yu, L. Shen, L. Yao, N. Singh, Discovery of a novel protein kinase B inhibitor by structure-based virtual screening, *Bioorg Med Chem Lett*, 19 (2009) 4634-4638.
- [54] H. Choi, H.J. Park, J.C. Shin, H.S. Ko, J.K. Lee, S. Lee, H. Park, S. Hong, Structure-based virtual screening approach to the discovery of p38 MAP kinase inhibitors, *Bioorg Med Chem Lett*, 22 (2012) 2195-2199.
- [55] S. Li, X. Sun, H. Zhao, Y. Tang, M. Lan, Discovery of novel EGFR tyrosine kinase inhibitors by structure-based virtual screening, *Bioorg Med Chem Lett*, 22 (2012) 4004-4009.
- [56] L. Di-Wu, L.L. Li, W.J. Wang, H.Z. Xie, J. Yang, C.H. Zhang, Q. Huang, L. Zhong, S. Feng, S.Y. Yang, Identification of CK2 inhibitors with new scaffolds by a hybrid virtual screening approach based on Bayesian model; pharmacophore hypothesis and molecular docking, *J Mol Graph Model*, 36 (2012) 42-47.
- [57] M.I. Davis, J.P. Hunt, S. Herrgard, P. Ciceri, L.M. Wodicka, G. Pallares, M. Hocker, D.K. Treiber, P.P. Zarrinkar, Comprehensive analysis of kinase inhibitor selectivity, *Nat Biotechnol*, 29 (2011) 1046-1051.
- [58] B.D. Marsden, S. Knapp, Doing more than just the structure-structural genomics in kinase drug discovery, *Curr Opin Chem Biol*, 12 (2008) 40-45.

- [59] J. Eswaran, S. Knapp, Insights into protein kinase regulation and inhibition by large scale structural comparison, *Biochim Biophys Acta*, 1804 (2010) 429-432.
- [60] M.J. Schnieders, T.S. Kaoud, C. Yan, K.N. Dalby, P. Ren, Computational insights for the discovery of non-ATP competitive inhibitors of MAP kinases, *Curr Pharm Des*, 18 (2012) 1173-1185.
- [61] M.A. Fabian, W.H. Biggs, 3rd, D.K. Treiber, C.E. Atteridge, M.D. Azimioara, M.G. Benedetti, T.A. Carter, P. Ciceri, P.T. Edeen, M. Floyd, J.M. Ford, M. Galvin, J.L. Gerlach, R.M. Grotzfeld, S. Herrgard, D.E. Insko, M.A. Insko, A.G. Lai, J.M. Lelias, S.A. Mehta, Z.V. Milanov, A.M. Velasco, L.M. Wodicka, H.K. Patel, P.P. Zarrinkar, D.J. Lockhart, A small molecule-kinase interaction map for clinical kinase inhibitors, *Nat Biotechnol*, 23 (2005) 329-336.
- [62] S.K. De, L.H. Chen, J.L. Stebbins, T. Machleidt, M. Riel-Mehan, R. Dahl, V. Chen, H. Yuan, E. Barile, A. Emdadi, R. Murphy, M. Pellecchia, Discovery of 2-(5-nitrothiazol-2-ylthio)benzo[d]thiazoles as novel c-Jun N-terminal kinase inhibitors, *Bioorg Med Chem*, 17 (2009) 2712-2717.
- [63] R.K. Hom, S. Bowers, J.M. Sealy, A.P. Truong, G.D. Probst, M.L. Neitzel, R.J. Neitz, L. Fang, L. Brogley, J. Wu, A.W. Konradi, H.L. Sham, G. Toth, H. Pan, N. Yao, D.R. Artis, K. Quinn, J.M. Sauer, K. Powell, Z. Ren, F. Bard, T.A. Yednock, I. Griswold-Prenner, Design and synthesis of disubstituted thiophene and thiazole based inhibitors of JNK, *Bioorg Med Chem Lett*, 20 (2010) 7303-7307.
- [64] J.C. Lee, J.T. Laydon, P.C. McDonnell, T.F. Gallagher, S. Kumar, D. Green, D. McNulty, M.J. Blumenthal, J.R. Heys, S.W. Land vatter, J.E. Strickler, M.M. McLaughlin, I.R. Siemens, S.M. Fisher, G.P. Livi, J.R. White, J.L. Adams, P.R. Young, A protein kinase involved in the regulation of inflammatory cytokine biosynthesis, *Nature*, 372 (1994) 739-746.

- [65] L. Tong, S. Pav, D.M. White, S. Rogers, K.M. Crane, C.L. Cywin, M.L. Brown, C.A. Pargellis, A highly specific inhibitor of human p38 MAP kinase binds in the ATP pocket, *Nat Struct Biol*, 4 (1997) 311-316.
- [66] S. Fischer, S.C. Koeberle, S.A. Laufer, p38alpha mitogen-activated protein kinase inhibitors, a patent review (2005-2011), *Expert Opin Ther Pat*, 21 (2011) 1843-1866.
- [67] A. Cuadrado, A.R. Nebreda, Mechanisms and functions of p38 MAPK signalling, *Biochem J*, 429 (2010) 403-417.
- [68] D.T. Rosenthal, H. Iyer, S. Escudero, L. Bao, Z. Wu, A.C. Ventura, C.G. Kleer, E.M. Arruda, K. Garikipati, S.D. Merajver, p38gamma promotes breast cancer cell motility and metastasis through regulation of RhoC GTPase, cytoskeletal architecture, and a novel leading edge behavior, *Cancer Res*, 71 (2011) 6338-6349.
- [69] F. Meng, H. Zhang, G. Liu, B. Kreike, W. Chen, S. Sethi, F.R. Miller, G. Wu, p38gamma mitogen-activated protein kinase contributes to oncogenic properties maintenance and resistance to poly (ADP-ribose)-polymerase-1 inhibition in breast cancer, *Neoplasia*, 13 (2011) 472-482.
- [70] A. Kukkonen-Macchi, O. Sicora, K. Kaczynska, C. Oetken-Lindholm, J. Pouwels, L. Laine, M.J. Kallio, Loss of p38gamma MAPK induces pleiotropic mitotic defects and massive cell death, *J Cell Sci*, 124 (2011) 216-227.
- [71] S.P. Davies, H. Reddy, M. Caivano, P. Cohen, Specificity and mechanism of action of some commonly used protein kinase inhibitors, *Biochem J*, 351 (2000) 95-105.

Table 1: Evaluation of JD123 as an inhibitor of MAPKs. Protein kinase assays, including JD123 or JD124, were performed under standardized conditions (National Centre for Protein Kinase Profiling, MRC Protein Phosphorylation Unit, University of Dundee). Substrate conditions [42, 71] are indicated, including the use of either 0.33mg/ml myelin basic protein (MBP) or 3 μ M ATF2 peptide [19-96]. Results (% remaining activity $\pm$ SEM) are provided for the 9 different MAPKs.

MAP K	Substrate conditions		% Activity remaining		
	[ATP] μ M	Protein	JD123		JD124
			10 μ M	100 μ M	100 μ M
p38 α	50	MBP	91 $\pm$ 1	78 $\pm$ 8	36 $\pm$ 2
p38 β	20	MBP	96 $\pm$ 7	87 $\pm$ 10	69 $\pm$ 29
p38 γ	5	MBP	15 $\pm$ 2	9 $\pm$ 1	60 $\pm$ 1
p38 δ	5	MBP	88 $\pm$ 2	46 $\pm$ 7	65 $\pm$ 17
JNK1	20	ATF2	81 $\pm$ 13	49 $\pm$ 0	83 $\pm$ 2
JNK2	20	ATF2	90 $\pm$ 7	64 $\pm$ 3	88 $\pm$ 0
JNK3	50	ATF2	70 $\pm$ 3	44 $\pm$ 4	80 $\pm$ 0
ERK1	5	MBP	66 $\pm$ 18	21 $\pm$ 12	19 $\pm$ 4
ERK2	50	MBP	93 $\pm$ 5	95 $\pm$ 15	73 $\pm$ 15

Table 2: Results of Kinase Inhibitor Profiling. Protein kinase assays, including JD123 (10 μ M), were performed under standardized conditions (National Centre for Protein Kinase Profiling). Results (% remaining activity $\pm$ SEM) are provided for 100 kinases for the most potently inhibited (columns 1 & 2) and least potently inhibited (columns 7 & 8).

Kinase	% Activity remaining	Kinase	% Activity remaining	Kinase	% Activity remaining	Kinase	% Activity remaining
PKB β	3 $\pm$ 1	PHK	54 $\pm$ 4	MELK	73 $\pm$ 9	TAO1	89 $\pm$ 4
PIM3	17 $\pm$ 2	Aurora A	54 $\pm$ 2	TrkA	73 $\pm$ 2	PAK2	89 $\pm$ 2
CLK2	18 $\pm$ 13	IKK β	56 $\pm$ 0	IRAK4	74 $\pm$ 1	CHK1	90 $\pm$ 7
CAMK1	20 $\pm$ 3	CHK2	57 $\pm$ 6	PAK4	74 $\pm$ 4	MEKK1	90 $\pm$ 2
PIM1	22 $\pm$ 3	HIPK3	60 $\pm$ 1	TBK1	75 $\pm$ 7	PKA	91 $\pm$ 9
MAPKAPK3	23 $\pm$ 4	HIPK2	61 $\pm$ 1	MST2	76 $\pm$ 9	PKB α	91 $\pm$ 4
BTK	26 $\pm$ 5	CAMKKb	62 $\pm$ 2	PRK2	76 $\pm$ 4	Src	91 $\pm$ 5
DYRK1A	33 $\pm$ 2	PIM2	63 $\pm$ 1	YES1	77 $\pm$ 3	MAPKAP K2	92 $\pm$ 10
IR	34 $\pm$ 1	CK2	63 $\pm$ 4	EF2K	77 $\pm$ 3	EPH-B2	93 $\pm$ 12
PLK1	35 $\pm$ 3	JAK2	63 $\pm$ 10	TAK1	79 $\pm$ 5	EPH-B1	93 $\pm$ 0
MINK1	35 $\pm$ 4	GCK	63 $\pm$ 0	ROCK2	80 $\pm$ 7	MKK6	94 $\pm$ 7
IRR	40 $\pm$ 1	CDK2-Cyclin A	63 $\pm$ 1	BRSK1	80 $\pm$ 7	Lck	94 $\pm$ 4
NUAK1	42 $\pm$ 7	LKB1	64 $\pm$ 2	MKK1	80 $\pm$ 6	PRAK	94 $\pm$ 11
NEK2a	42 $\pm$ 4	CK1	64 $\pm$ 1	MARK1	82 $\pm$ 12	PKC ζ	95 $\pm$ 6
HER4	43 $\pm$ 2	S6K1	64 $\pm$ 5	SRPK1	82 $\pm$ 12	MNK1	95 $\pm$ 4
DYRK3	45 $\pm$ 0	MNK2	65 $\pm$ 2	MARK4	83 $\pm$ 5	MST4	96 $\pm$ 6
VEGFR	45 $\pm$ 1	MLK1	65 $\pm$ 3	CSK	83 $\pm$ 3	SGK1	96 $\pm$ 6
FGFR1	47 $\pm$ 5	PKC α	67 $\pm$ 2	AMPK	83 $\pm$ 1	ASK1	96 $\pm$ 1
MLK3	47 $\pm$ 1	IKK ϵ	67 $\pm$ 10	HIPK1	84 $\pm$ 7	EPH-B4	97 $\pm$ 3
GSK3 β	48 $\pm$ 9	SmMLCK	68 $\pm$ 8	MARK3	84 $\pm$ 2	SYK	100 $\pm$ 2
MARK2	48 $\pm$ 2	BRSK2	70 $\pm$ 9	PDK1	84 $\pm$ 6	EPH-B3	101 $\pm$ 11
RSK2	50 $\pm$ 3	AuroraB	70 $\pm$ 3	PAK6	84 $\pm$ 8	EPH-A2	103 $\pm$ 10
DYRK2	50 $\pm$ 0	IGF-1R	70 $\pm$ 10	PKD1	86 $\pm$ 5	PAK5	103 $\pm$ 7
RIPK2	52 $\pm$ 1	DAPK1	71 $\pm$ 0	MKK2	87 $\pm$ 1	RSK1	105 $\pm$ 5
TTK	53 $\pm$ 8	NEK6	73 $\pm$ 0	MSK1	88 $\pm$ 14	EPH-A4	106 $\pm$ 5

Figure Legends

Figure 1. Representations of the JIP1/substrate docking site of JNK1. (A) Using the available structure of JNK1 α 1 bound to TIIP (Protein Data Bank (PDB): 1ukh) [32], the JIP1/substrate docking site of JNK1 was defined. This JNK1 surface was generated using GOLD (v.3.2) and all acidic (red) and basic residues (blue) identified. For simplicity, the TIIP peptide is shown in stick representation (green) with critical residues ¹⁵⁶Arg, ¹⁵⁷Pro, ¹⁶⁰Leu and ¹⁶²Leu denoted. (B) The JNK1 structure was further prepared for docking by excising the TIIP peptide and adjusting the protonation states of its amino acids to their states at pH 7.0. This region was further subdivided and subsequent docking studies were focused on the JNK1 region interacting with ¹⁵⁶Arg-¹⁵⁷Pro (referred throughout as the “RP site”). This JNK1 “RP site” is characterized by a shallow hydrophobic pocket and a series of acidic (Glu329, Asp326) and polar (Tyr130, Tyr133) residues that interact with the ¹⁵⁶Arg side-chain within TIIP.

Figure 2. Validation of JNK inhibitory molecules identified by virtual screening. (A) Active JNK1 α 1 and GST-cJun (1-135) were pre-incubated (5 min, 30°C) with selected candidate compounds (JD117, JD118, JD119, JD122, JD123, JD124, JD127; 100 μ M) identified in the virtual screening of commercially available chemical libraries. The entire range of compounds tested is summarized in Supplementary Table 1. Incubations including vehicle (V, 0.7% DMSO), or 2 μ M TIIP were included as negative and positive controls, respectively. JNK activity was estimated by quantitating the ³²P incorporation into GST-cJun (1-135) in three independent experiments (upper panel). Autoradiography indicated the phosphorylation level of cJun substrate (lower panel). Results were expressed as % of the uninhibited JNK1 activity; error bars represent the standard error of the means and asterisks indicate values statistically significantly different (*, p<0.05; **, p<0.01; n=3) from the non-

inhibited control reactions. The results for the JD series of compounds have been presented in order of potency of JNK inhibitory activity observed under these assay conditions. (B) Chemical structures of the tested candidate compounds (JD117, JD118, JD119, JD122, JD123, JD124, JD127).

Figure 3. Both molecular modeling and kinetic analyses reveals JD123 as a dual inhibitor of JNK1. (A) The structure of neutral and protonated forms of JD123 (4'-methyl-N²-3-pyridinyl-4,5'-bi-1,3-thiazole-2,2'-diamine; Chembridge ID: 7850865). (Bi) The docking poses of the neutral (top) and protonated (bottom) forms of JD123 in the JNK1 “RP site”. The top panel illustrates the 3 clusters of poses observed when the neutral form of the molecules was docked, while the bottom panel illustrates the single pose observed when the protonated form of the ligand was used in the docking. (Ci) The docking poses of JD123 in the ATP-binding pocket of JNK1. The JNK1 surface was generated using GOLD (v.3.2) and all acidic (red) and basic (blue) residues identified. (Bii) and (Cii) Kinetic analysis of the effects of JD123 (10 – 40 μ M) to inhibit JNK1 activity towards cJun was performed. In these assays, the level of ³²P incorporation into the GST-cJun (1-135) substrate was quantitated. Data was analysed by Lineweaver-Burk plot ((1/v (min/pmol) vs 1/[cJun] or 1/[ATP] (μ M⁻¹)) and linear regression analyses. (Ci) The inhibition of JNK1 towards a range of GST-cJun (1-135) concentrations (0.8 - 8.1 μ M) by the various JD123 concentrations was evaluated at a fixed ATP concentration (20 μ M). (Cii) The inhibition of JNK1 activity by the various JD123 concentrations at a fixed concentration of GST-cJun (1-135) (8.1 μ M) was examined with a range of ATP concentrations (2 – 20 μ M).

Figure 4. STD NMR experiment confirms JD123 targeting JNK1 at both ATP and substrate-binding sites. Competition STD NMR spectra of 300 μ M samples of AMP-PNP

and TIJIP with JD123 in 10 μ M JNK1, pH 6.8 and 25 °C. The residual water line has been removed from each spectrum. (A) Reference spectrum of AMP-PNP with JNK1. The resonances from the H7 and H12 protons of the adenine ring, and the H2' of the ribose group of AMP-PNP are annotated. (B) STD NMR spectrum showing STD enhancement signals of AMP-PNP. (C) STD NMR spectrum of AMPPNP and JD123 with JNK1. STD enhancement signals of JD123 were observed, whereas no signals for AMP-PNP were observed. The five aromatic resonances of JD123 were observed in the downfield portion of the spectrum, and a resonance corresponding to the methyl is indicated in the upfield part of the spectrum. (D) Reference spectrum of TIJIP with JNK1. (E) STD NMR spectrum showing STD enhancement signals of TIJIP. (F) STD NMR spectrum of TIJIP and JD123 with JNK1. STD enhancement signals of JD123 were observed, and the STD signals for TIJIP were reduced.

Figure 5. JD123 dose-dependently inhibits JNK1 phosphorylation towards various substrate proteins. Active JNK1 α 1 and its substrates (A, GST-cJun (1-135); B, GST-ATF2 (19-96); C, GST-Elk1 (307-428); or D, GST-DCX (1-366)) were pre-incubated (5 min, 30°C) in the presence of 10, 40 and 100 μ M JD123 (4'-methyl-N²-3-pyridinyl-4,5'-bi-1,3-thiazole-2,2'-diamine; Chembridge ID: 7850865), before assaying the kinase activity of JNK1. TIJIP (2 μ M) was included as a positive control. The level of ³²P incorporation into each JNK substrate was quantitated and the results were expressed as % of the uninhibited kinase activity in three independent experiments (upper panel). Autoradiography indicated the phosphorylation level of each substrate (lower panel). Error bars represent the standard error of the means and asterisks indicate values statistically significantly different (*, $p < 0.05$; **, $p < 0.01$; $n = 3$) from the non-inhibited controls.

Figure 6. JD123 can inhibit cJun Ser63 phosphorylation following hyperosmotic stress.

Murine embryonic fibroblasts (MEFs) were pre-treated with DMSO as vehicle control, JD123, or JD124 as negative control, before being subjected to hyperosmotic stress (0.5M sorbitol; 60min). (A) Protein lysates were prepared and blotted with antibodies to phosphoSer63 cJun, total cJun, phosphoJNK1/2 and total JNK1/2. Experiments were repeated on 3 independent occasions with comparable results. (B) Quantitation and data analysis from triplicate experiments of JD123 inhibition were expressed as % of p-cJun S63 signal. Error bars represent the standard error of the means and asterisks indicate values statistically significantly different (**, $p < 0.05$; $n = 3$) from the non-inhibited controls.

Figure 7. JD123 inhibition of JNK1 towards the EGFR-based peptide substrate.

Active JNK1 and EGFR-based peptide substrate (sequence: KRELVEPLTPSGEA) were pre-incubated with the different inhibitors specified. Several controls were included in the assay, including ATP-competitive JNK inhibitor VIII (positive control), ATP-noncompetitive inhibitor TIJIP (negative control), DMSO (V, vehicle; at final concentrations of 1, 4 and 10% equivalent to the levels included in the assays with JD123) or JD123 (10, 40 and 100 μ M). The level of 32 P incorporation into the peptide substrate in each reaction was quantitated by liquid scintillation counting and the results were expressed as % of the uninhibited kinase activity in three independent experiments. Error bars represent the standard error of the means and asterisks indicate values statistically significantly different (**, $p < 0.01$; $n = 3$) from the non-inhibited controls.

Figure 8. Effect of JD123 on the activity of other MAPKs.

(A) Active ERK1 and GST-Elk1 (307-428), (B) active ERK2 and GST-Elk1 (307-428), and (Ci) active p38 γ and GST-ATF2 (19-96) were pre-incubated with JD123 (10, 40 and 100 μ M) before assaying the

kinase activity *in vitro*. For each set of results, autoradiography indicated the level of phosphorylation of substrates (lower panels), and the incorporation of ^{32}P into each substrate was quantified by liquid scintillation counting (upper panels). Results from three independent experiments were expressed as % of the uninhibited kinase activity, and error bars represent the standard error of the means and asterisks indicate values statistically significantly different (**, $p < 0.01$; $n = 3$) from the non-inhibited controls. (Cii), Kinetic analysis of JD123 (0 – 10 μM) to inhibit p38 γ activity towards ATF2 was assessed by examining the effect of various JD123 concentrations at a fixed concentration of GST-ATF2 (19-96) (8.1 μM) at a range of ATP concentrations (2 – 20 μM).

A **B** **Figure 1**

Figure 2

Figure 3

Figure 4

Figure 5

A **B**

Figure 6

Figure 7

Figure 8

Highlights

- Protein Kinases are critical enzymes in signal transduction networks
- Virtual screening identified bi-thiazole-2,2'-diamines as potential JNK inhibitors
- JNK was inhibited by the compound JD123, but not a related compound JD124
- JD123 inhibited the related kinase p38 γ ; broader screening showed Akt β inhibition
- Bi-thiazoles can act as a new protein kinase inhibitory scaffold

Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:

Ngoei, KRW;Ng, DCH;Gooley, PR;Fairlie, DP;Stoermer, M;Bogoyevitch, MA

Title:

Identification and characterization of bi-thiazole-2,2'-diamines as kinase inhibitory scaffolds

Date:

2013-06-01

Citation:

Ngoei, K. R. W., Ng, D. C. H., Gooley, P. R., Fairlie, D. P., Stoermer, M. & Bogoyevitch, M. A. (2013). Identification and characterization of bi-thiazole-2,2'-diamines as kinase inhibitory scaffolds. *BIOCHIMICA ET BIOPHYSICA ACTA-PROTEINS AND PROTEOMICS*, 1834 (6), pp.1077-1088. <https://doi.org/10.1016/j.bbapap.2013.02.001>.

Persistent Link:

<http://hdl.handle.net/11343/43891>