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A Small Molecule Bidentate-Binding Dual Inhibitor Probe of the LRRK2 and JNK Kinases

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Abstract

Both JNK and LRRK2 are associated with Parkinson's disease (PD). Here we report a reasonably selective and potent kinase inhibitor (compound **6**) that bound to both JNK and LRRK2 (a dual inhibitor). A bidentate-binding strategy that simultaneously utilized the ATP hinge binding and a unique protein surface site outside of the ATP pocket was applied to the design and identification of this kind of inhibitor. Compound **6** was a potent JNK3 and modest LRRK2 dual inhibitor with an enzyme IC₅₀ value of 12 nM and 99 nM (LRRK2-G2019S), respectively. **6** also exhibited good cell potency, inhibited LRRK2:G2019S induced mitochondrial dysfunction in SHSY5Y cells, and was demonstrated to be reasonably selective against a panel of 116 kinases from representative kinase families. Design of such a probe molecule may help enable testing if dual JNK and LRRK2 inhibitions have added or synergistic efficacy in protecting against neurodegeneration in PD.

Keywords

Dual Inhibitor; Bidentate-Binding kinase inhibitors; JNK3; LRRK2; Parkinson's Disease; PD

The design and identification of potent and highly selective JNK inhibitors has been avidly pursued in the past few years due to potential wide spread therapeutic applications.^{1–4} In particular, development of brain penetrant small molecule inhibitors for JNK and LRRK2 has been a major focus in order to develop efficacious therapeutics for Parkinson's disease (PD)^{2, 3, 5–10} and other neurodegenerative diseases for JNK, such as Alzheimer's (AD)¹¹, Huntington's disease (HD)¹², amyotrophic lateral sclerosis (ALS)¹³ and multiple sclerosis (MS).¹⁴ To this end, our lab and the Elan group have successfully developed selective, brain penetrant, and orally bioavailable small molecule JNK inhibitors^{2, 3, 15–19} which showed good efficacy for the treatment of neurodegeneration models in particular and PD animal models specifically (such as SR3306 developed in our labs). LRRK2 inhibitors have also been discovered in several labs,^{5, 7} however the selectivity, cell potency, and especially the brain penetration capability for these initial compounds still need improvement. Recent

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Supporting Information. Synthesis, characterization, and spectroscopy for compounds **3** to **8**; biochemical and cell assay protocols, methods of docking, profiling data against 96 kinases. This material is available free of charge via the Internet at <http://pubs.acs.org>

publications from Genentech^{8,9} and a review from Schmidt¹⁰ have highlighted a series of potent, highly selective, and brain penetrant aminopyrimidines that are excellent candidates as LRRK2 development candidates.

To extend our work in developing novel neuroprotective therapeutics for PD, we set out to discover unique JNK inhibitors from diversified scaffolds. It was our goal to develop compounds that were capable of inhibiting both JNK3 and LRRK2 simultaneously (dual inhibitors) in the hope that these compounds would exhibit greater efficacy than compounds that inhibited only JNK or LRRK2 individually. Dual inhibitors can be used as *in vitro* or *in vivo* probes to test the hypothesis that dual inhibition of JNK and LRRK2 may be additive or synergistic in the treatment of both familial and idiopathic PD. A dual inhibitor is preferred over combined, individual JNK and LRRK2 inhibitors because it eliminates complications of drug-drug interactions and the need to optimize individual inhibitor doses for efficacy.

The major challenge in developing kinase inhibitors is to gain high selectivity in order to diminish off-target side effects, which is especially important for non-oncogenic targets such as for CNS applications. Some type-II and type-III kinase inhibitors have given high selectivity since these compounds bind to protein pockets that are unique for a specific kinase, such as the allosteric site for type-III inhibitors²⁰ and the hydrophobic pocket occupied originally by the Phe residue in the DFG-in conformation for type-II inhibitors.²¹ Others, such as BIRB-796 have not been as selective as some type I inhibitors.¹⁸ Additionally, application of type-II and type-III inhibitors can be limited because many kinases cannot assume a DFG-out conformation and allosteric binding sites have been discovered for only a few kinases. The majority of kinase inhibitors developed so far are ATP-competitive and their selectivity can be low due to binding in the highly conserved ATP-binding pocket. Despite this, very selective type-I inhibitors have indeed been developed however^{22–26}.

Here we present a strategy for identifying small molecule kinase inhibitors that combine the advantages of both type-I inhibitors (for easy access to kinase inhibitors with high affinity) and type-II/III inhibitors (for high selectivity). Specifically, our strategy is to design *bidentate-binding inhibitors*^{27,28} that can simultaneously bind to the kinase hinge (mimicry of type-I inhibitors) and to a surface pocket close-by but outside of the hinge region and/or the ATP pocket (mimicry of type-II/III inhibitors). This surface pocket could be a substrate binding site or an allosteric binding pocket. Indeed, our group has recently reported the three dimensional structure of JNK3 solved with three different substrate peptides binding in the kinase interaction motif domain that is unique to JNK.²⁹ More importantly however, the site could be any surface pocket that can provide binding affinity for a small structural element. As long as the selected surface binding site is unique to a specific protein kinase, it is reasonable to assume that the resulting bidentate-binding inhibitors will exhibit high selectivity. Given the hydrophobic nature of the JIP binding pocket SAR plans will try to exploit this feature. Similarly, known interaction motifs in the ATP pocket such as crucial H-bonds and exploitation of the hydrophobic hole in the ATP pocket will be explored. A schematic representation for this bidentate binding strategy is demonstrated in Figure 1.

RESULTS AND DISCUSSION

For each c-jun N-terminal kinase (JNK) isoform, there exists a substrate-binding pocket in proximity to the hinge region of the ATP pocket.^{30–33} Several 11-mer peptides derived from its scaffolding proteins (JNK interacting proteins, JIP) have been demonstrated to be potent substrate-competitive JNK inhibitors (JIP-peptides).^{30–35} Due to the close-proximity of this substrate-binding site to the ATP pocket, Stebbins *et al* prepared a series of potent and

selective bidentate-binding JNK1/2 inhibitors composed of the 11-mer JIP-peptides and a hinge binder connected through various linkers.^{27, 28} After optimizations, they were able to reduce the size of the peptide portion from 11-mers to the tri-peptide LNL (compound **1**, Figure 1A).²⁷ Compound **1**, which utilized a 3-phenyl-indazole as the hinge binder and a moiety of propyl-1,3-diamine coupled with a di-Gly as the linker, still had good potency in both kinase activity assay and JIP displacement assay (Table 1, data were obtained in-house at Scripps). However, compound **1** and its analogs (with longer peptide moieties) are still peptide-like and possess all the major drawbacks associated with peptide-based drugs.

It was our belief that the JIP-site-binding tri-peptide moiety in **1** (Ac-LNL-) could be replaced by non-peptidic elements and the resulting bidentate inhibitors would potentially be more drug-like. A series of exploratory studies were embarked on to identify key molecular moieties. As shown in Figure 1, removal of the N-terminal acetyl group (**2**) reduced the JNK3 inhibition (Table 1). The linker length could be shortened from 12 backbone atoms (by replacing the propyl-1,3-diamine coupled with a di-Gly in **1** and **2**) to 10 atoms in **3** without hurting the JIP displacement activity (Table 1). Several optimization strategies were applied to reduce the peptidic nature of these bidentate inhibitors including addition of a benza-dioxane ring in lieu of the dipeptide (compound **4**). This change showed JNK3 inhibition activity and JIP displacement potency, within error, similar to that of **2** and **3** (Table 1). In addition, a peptoid strategy was used to modify one of the Leu residues and the middle Asn residue in the tri-peptide LNL was replaced simply by Gly. More interestingly, the terminal Leu residue could be substituted by a benzamide moiety. It was no surprise that all these modifications were possible since the tri-peptide, LNL, in **1** binds mainly to a hydrophobic pocket in the JIP site.²⁷ Interestingly, the linker length in **4** was further reduced to 9 backbone atoms and the Gly moiety was totally removed from the linker (Figure 1), further increasing the small molecule-like nature of the resulting bidentate kinase inhibitors.

The most exciting results were obtained when the terminal benzadioxane-6-carboxyl amide in **4** was replaced by its regio-isomer benzadioxane-2-carboxyl amide to give compound **5**, which had comparable JNK3 inhibition activity (and a slightly better potency for JNK1) and a better JIP FP displacement potency compared to compound **1**. Further optimizations on the terminal bicyclic amide produced the best bidentate-binding kinase inhibitor **6** (also coded as **SR9444**) for this series, where a chroman-3-carboxyl amide³⁶ was used to displace the benzadioxane amide. This modification was able to increase the potency (IC₅₀ value was 12 nM and 336 nM for JNK3 inhibition and JIP FP displacement, respectively, Table 1). Interestingly, replacement of the Gly in **6** by an Ala residue significantly reduced the potency in both JNK3 and JIP displacement assays (compound **7**). Moreover, a series of other hinge binding moieties were assessed (such as the 5-yl-indazole in compound **8**, Figure 1, Table 1) yet the 3-(4-yl-phenyl)indazole was discovered to be still the best.

The JIP-peptide displacement capability of designed bidentate inhibitors

As described above, the bidentate-binding property of compound **6** and its analogs was demonstrated by the displacement of JIP-peptide in JIP FP assay, and the strong inhibition of JNK activity in the enzyme activity assay (Table 1, JNK inhibition activity). The biochemical assay for JIP displacement is centered on utilizing a TAMRA-JIP-11-mer peptide (TAMRA-RPKRPTTLNLF) with JNK3 39–422 and measuring fluorescence polarization (FP) changes in the presence and absence of small molecule inhibitors. This assay has been established in 96-well and 384-well format in our labs. Unlabeled JIP-peptide (RPKRPTTLNLF) and compound **2** from Chen *et al.*³⁷ was used as positive controls. The dose-response curve shown in Figure 2 clearly demonstrated the ability of compound **6** to displace the JIP-peptide with an IC₅₀ value of ~ 336 nM.

The detection method in the JNK3 enzyme assay allows for the detection of a compound that inhibits by a variety of mechanisms including: substrate-site binding, allosteric site binding, and ATP-competitive binding. Assays shown in Figure 2 already demonstrated the substrate site binding property of compound **6**. However, if the inhibition of kinase activity by **6** was only through substrate site binding, its IC₅₀ value in JNK3 activity assay should be similar to that in JIP displacement assay. As shown in Table 1, compound **6** (and its analogs) exhibited a much higher potency in JNK3 activity assay than in the JIP displacement assay (12 nM vs. 336 nM), indicating the existence of other interactions in addition to JIP substrate site binding. One possible interaction besides JIP site binding was ATP hinge binding because the head indazole moiety is a well-known hinge binder.^{38,39} Indeed, mechanism of inhibition studies revealed that compound **6** was a competitive inhibitor of ATP showing non-linear fits that are reflective of competitive inhibition and Lineweaver-Burk plots showing the intersecting line pattern representative of an ATP competitive inhibitor (Supplemental Figure 1). Data were fit to equations for competitive, non-competitive, uncompetitive, and mixed inhibition. χ^2 analysis and F-test goodness of fit revealed competitive inhibition for compound **6** ($p < 0.05$).

Synthesis of bidentate inhibitors

Synthesis of compound **6** is presented in Scheme 1. A Suzuki coupling between compounds **9** and **10** was used to give 3-(4'-carboxylphenyl)indazole **11**. An S_N2 reaction between compound **12** and isobutylamine was applied to yield secondary amine **13**, followed by amide formation with Boc-Gly using EDC and HOBt as the coupling reagents to produce **14**. After removal of the *N*-Boc-protection using TFA in dichloromethane, another amide coupling was carried out to give methyl ester **15**. Aqueous hydrolysis of **15** using KOH followed by amide formation with *N*-Boc-hexane-1,6-diamine resulted in compound **16**. Finally, Boc-removal by TFA and amide formation with compound **11** yielded compound **6**, which was purified by reverse-phase prep HPLC. Similar procedures were also applied to synthesize other compounds in Figure 1, which were all characterized by NMR and MS.

Kinase selectivity of bidentate inhibitors

Our initial enzyme inhibition profiling against a panel of 21 representative kinases from several kinase families (JNK3, JNK1, p38 α , ROCK1, ROCK2, CDK5, CDK7, CLK1, EGFR, ERK2, FLT1, GSK3 α , IKK β , JAK3, LCK, LIMK1, LRRK2, MKK4, PKA, SGK1, and SYK) at 10 μ M inhibitor concentration, revealed that compound **6** inhibited significantly only JNKs (~100%) and LRRK2 (94%), and had moderate inhibition of CLK1 (50%, i.e., IC₅₀ ~10 μ M). To augment this we screened an additional 96 kinases at 10 μ M in the Ambit binding screen²⁶ to get a broader sense for the selectivity of this compound. Supplemental Table 1 and supplemental Figure 1 show that only six of the 96 kinases tested had greater than 90% binding to **6** at 10 μ M. Given the high concentration used in this assay, the data suggest that **6** is a largely selective JNK and LRRK2 kinase inhibitor (~ 5% of kinases inhibited by **6**) and that this selectivity could possibly be attributed to the bidentate binding nature of **6**. Since minimal SAR optimizations have been made on this probe molecule it is believed that high selectivity for this class of inhibitors could easily be achieved by exploiting the bidentate binding character of this class.

Inhibition of designed bidentate inhibitor against LRRK2

The strong inhibition of **6** against LRRK2 was a surprise but also a pleasant bonus for our efforts since we hypothesized that dual JNK3 and LRRK2 inhibition may be more efficacious in neuroprotection, especially since both targets have demonstrated benefit in PD models, and a dual inhibitor is what we are searching for. To demonstrate the ability of LRRK2 inhibition, compound **6** was titrated in both wild-type LRRK2 and PD specific

mutant LRRK2-G2019S (Figure 3), and was found to possess an enzyme inhibition activity of $IC_{50} \sim 100$ nM for both of them. High LRRK2 inhibition was also found for most of the bidentate inhibitors with similar structures to that of compound **6** (data not shown), indicating that there is a general dual inhibition pattern for this series.

Docking studies of compound **6** in JNK3 and LRRK2

While a co-crystal structure of **6** with JNK3 (an endeavor which is under way in our lab) is required to pinpoint the molecular interactions that describe the binding motif of these JNK/LRRK2 dual inhibitors, docking studies of **6** with both JNK3 and LRRK2 were performed for compound **6** as a surrogate in order to provide binding modes to guide further optimizations. Thus, compound **6** was docked into the X-ray crystal structure of JNK3 39–402 and the JIP peptide²⁹ using Glide SP v5.8 (Schrodinger, LLC, NY). Figure 4A presents the structure of JNK showing the JIP and ATP binding pockets and serves as an orientation and comparison for the modeling of compound **6** presented in Figure 4B. As shown in Figure 4B, the indazole-phenyl moiety of **6** H-bonds to the hinge with the benzamide pointing toward the catalytic loop and the solvent. The docking mode exhibited in Figure 4B revealed that the tail part of **6** bound to two sub-pockets of the JIP binding site in JNK3, with the N-isobutyl side chain binding to pocket-1 and the chroman ring binding to pocket-2. The two sub-pockets were composed mostly of hydrophobic residues which were responsible for recognizing JIP through a highly conserved $(R/K)(X)_4(L/V)XL$ motif. Sub-pocket-1 bound conserved Leu/Val whereas sub-pocket-2 holds the aliphatic side chain of the Leu/Arg/Lys residues. It is interesting to note that similar hydrophobic interactions occurred between JNK3 and compound **6**, although H-bonding interactions were also observed. Thus, this binding motif explains why **6** could displace the JIP-peptide with an IC_{50} value of 336 nM (Table 1), despite utilizing binding energy from less than half of the JIP binding sites.

Compound **6** was docked into a homology model of human LRRK2 by using Phyre2, a web based server to model human LRRK2 kinase domain (Figure 4C). The server produced 20 models with 100 % confidence where the sequence alignment between template and query ranged from 22 to 30%. We retrieved six top ranked models and comparison of the models showed an average r.m.s.d. of 1.1 Å over core kinase domains indicating the similarity among the models. We further inspected the models using PyMol to inspect the hinge binding region and the adjacent surface binding pockets. We chose the top ranked model based on the crystal structure of c-Abl Tyrosine Kinase with the PDB ID of 2FO0 (sequence identity of 23 % with human LRRK2) for the docking experiment. Importantly, the chosen model structure had hydrophobic pockets similar to the JIP binding site of JNK3. As shown in Figure 4C, the indazole moiety bound, as expected, to the hinge with H-bond formation. The homology model of human LRRK2 showed similar surface binding pockets to JIP binding sites in JNK3 at the C-lobe near the ATP pocket. Interestingly, the tail part of the inhibitor bound to surface pockets in an orientation very similar to that in JNK3. This surface-pocket-binding was reinforced by strong hydrophobic interactions of the isobutyl group and the chroman ring in their corresponding binding pockets. Finally, our model showed similarity to key residues highlighted by Chen et al.⁸ especially in the ATP pocket which was favored in the Chen study, but also revealed some differences due to the focus of our work on the hydrophobic pocket outside the ATP domain.

Cell activities of compound **6**

To analyze the cell activity of compound **6** and its analogs an In-cell Western detection assay was set up where phosphorylation of c-Jun was monitored. As seen in Figure 5, compound **6** began to inhibit the phosphorylation significantly from ~ 100 nM and its IC_{50} was calculated to be 2.8 μ M, indicating that **6** is a cell permeable small molecule inhibitor.

This finding was corroborated by Western blot analysis in H9C2 and N2a cells (Supplemental Figure 2). Considering the reasonably high biochemical kinase selectivity (coupled with its low μM cell potency), inhibitor **6** is believed to be a good *in vitro* probe molecule for studying the biology and the signal pathways related to JNK/LRRK2 (and mutant LRRK2s).

We next wanted to test if compound **6** was potent in cell-based functional assays that measured reactive oxygen species (ROS) generation, mitochondrial membrane potential (MMP), and cell viability. We had previously shown that JNK inhibition had significant effects on all of these mitochondrial functional parameters in HeLa cells.^{40,41} Figure 6 presents the effects that compound **6** had on all of these measures in the human dopaminergic SHSY5Y cell line. Figure 6A shows that transfection of SHSY5Y cells with LRRK2:G2019S caused a ~ 2-fold increase in ROS generated as measured by Mitosox fluorescence. Addition of either 1 μM or 10 μM compound **6** reduced the ROS levels in a statistically significant manner ($p < 0.05$). Similarly, compound **6** protected against the LRRK2:G2019S-induced decreases in MMP in a dose dependent manner where 10 μM compound **6** returned MMP dissipation to untreated levels (Figure 6B). Finally, LRRK2:G2019S-induced cell loss was measured showing ~ 50% less viable cells (Figure 6C). In the presence of 10 μM compound **6**, cell viability returned to > 95% ($p < 0.05$). These results indicate that the bidentate dual inhibitor **6** is effective against representative models of LRRK2:G2019S-induced toxicities in human dopaminergic cells.

Although compound **6** is a good *in vitro* probe (Table 1, Figures 2,3,5,6), its cell penetration capability still needs improvement. Compared to its potent JNK3 enzyme inhibition (IC_{50} : 12 nM), the cell potency of **6** (IC_{50} : 2.8 μM) had a right shift of > 200-fold, indicating a necessity for improvement in cell permeability. The high amide bond counts, the large PSA value (155), and the relatively high molecular weight (697) for **6** most likely will affect its cell permeability, and these properties will be the focus of future optimizations to obtain improved LRRK2/JNK3 dual inhibitors. Moreover, in order to develop dual inhibitors for probing PD and other CNS and non-CNS diseases *in vivo*, structural modifications will be required to obtain dual inhibitors which possess appropriate DMPK properties suitable for an administration route other than the intracerebroventricular or topical administrations.

In summary, we have successfully developed non-peptide small molecule bidentate dual inhibitors for JNK3/LRRK2. More importantly, this bidentate-binding design strategy, which combines binding to both the ATP hinge area and a protein surface pocket, could find wide applications in the development of highly selective and potent kinase inhibitors as probe molecules, since surface pockets close to the hinge area exist in almost all kinases. In addition, in the design of a bidentate kinase inhibitor, there may be no need to use a strong hinge binder and/or a strong-binding fragment for the protein surface pockets (indicating that the key binding moieties could be very small, and thus the whole inhibitor molecule may not have a very high MW). The synergy effects resulting from the simultaneous binding to multiple pockets should produce a strong protein modulator composed of weaker-binding structural fragments, provided the fragments are covalently connected with an appropriate linking moiety. More bidentate-binding kinase inhibitors and SAR studies for the optimization of compound **6** are under way in our labs. For example, to reduce the inhibitor size (or molecular weight), bidentate compounds that bind only to pocket-1 in both LRRK2 and JNK3 (Figure 4) will be designed and evaluated. These SAR research will be reported in subsequent manuscripts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

JNK	c-jun N-terminal kinase
LRRK2	Leucine rich repeat kinase 2
DCM	dichloromethane
DMF	N,N-dimethylformamide
EDC	ethyl-N,N-dimethylaminopropylcarbodi-imide
HOBt	N-hydroxybenzotriazole
TFA	trifluoroacetic acid
DIEA	diisopropylethylamine
Boc	<i>tert</i> -butoxycarbonyl
HPLC	high pressure liquid chromatography
MS	mass spectroscopy
NMR	nuclear magnetic spectroscopy
PSA	polar surface area
CNS	central nervous system
DMPK	drug metabolism and pharmacokinetics

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Figure 1. (A), evolution from peptide to small molecular bidentate-binding JNK3 inhibitors. (B), a schematic representation for the binding of these designed bidentate kinase inhibitors.

Figure 2.
JIP FP displacement assays for compound **6**.

Figure 3.
Enzyme assays for compound 6 in A: wild-type LRRK2 (IC₅₀: 118 nM); B: LRRK2-G2019S (IC₅₀: 99 nM)

Figure 4.

Ribbon representation of the crystal structure of JNK3 in complex with AMP-PCP (PDB ID 1JNK) (A). JIP peptide derived from PDB ID 4H39 is placed to show JIP binding pockets, Pocket1, and Pocket2. N-lobe and C-lobe are colored wheat and light blue, respectively. Ligand binding pockets are shown as a transparent surface with yellow and cyan respectively representing hydrophobic and polar regions. Only the boxed area of each protein is shown in panels B and C. Docking poses of compound 6 to the crystal structure of human JNK3 (B) and a homology model of LRRK2 (C) are shown in sticks with the ligand

binding pockets overlaid with transparent surface. Key residues involved in H-bond interactions with **6** are labeled. H-bonds are shown as dashed lines.

Figure 5.

Inhibition of the c-jun phosphorylation by compound **6** in H9C2 cardiomyocyte cells. The IC_{50} was calculated to be $2.8 \pm 0.5 \mu M$. Standard error is given.

Figure 6.

Protection of LRRK2:G2019S-induced mitochondrial dysfunction and cell death by compound **6**. Significance between LRRK2:WT and LRRK2:G2019S are indicated by a single asterisk ($p < 0.05$) (*), while differences between cells expressing LRRK2:G2019S and compound **6** treated cells expressing LRRK2:G2019S are indicated by a double asterisk ($p < 0.05$) (**). Error bars denote standard deviation.

^a Reagents and conditions: (a) Pd[P(Ph)₃]₄ (10%), K₂CO₃ (5 equiv), **9** (1 equiv), **10** (1.1 equiv), dioxane/H₂O (4:1), 95 °C, 4 h. (b) **12** (1 equiv), isobutylamine (1.1 equiv), THF, r.t., 2 h. (c) **13** (1 equiv), Boc-Gly (2 equiv), EDC/HOBt (2 equiv), DIEA (4 equiv), DMF, r.t., overnight. (d) 33% TFA in DCM, r.t., 1 h. (e) acid (1.1 equiv), EDC/HOBt (1.2 equiv), DIEA (2 equiv), DMF, r.t., 2 h. (f) KOH (2 equiv), THF, r.t., 3 h. (g) N-Boc-1,6-hexanediamine (1.2 equiv), EDC/HOBt (1.2 equiv), DIEA (2 equiv), DMF, r.t., 2 h. (h) **11** (1.1 equiv), EDC/HOBt (1.2 equiv), DIEA (2 equiv), DMF, r.t., 2 h.

Table 1

Enzyme assay data for bidentate inhibitors

Compd	JNK3 IC ₅₀ (nM) ^a	JNK1 IC ₅₀ (nM) ^a	JIP FP displacement IC ₅₀ (nM) ^a
1	37.8 ± 11.8	n.d	760
2	135.5 ± 35.9	n.d	624
3	157.5 ± 161.6	317.9 ± 11.9	620
4	147.4 ± 51.6	684.6 ± 121.4	907
5	63.4 ± 10.4	164.4 ± 21.7	363
6	11.6 ± 2.4	109.4 ± 6.1	336
7	126.7 ± 30.3	154.5 ± 37.1	1045
8	3154	n.d	n.d
SP600125^b	220 ± 42	68.0 ± 10.3	n.d

^aIC₅₀ values were calculated from 2–3 determinations.^bSP600125 was used as the positive control JNK inhibitor in our enzyme assays. Data were determined from 10 measurements.