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A Fragment-Based Approach for the Discovery of Isoform-Specific p38α Inhibitors

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ABSTRACT In this study, we describe a novel approach for lead discovery against protein kinases, pharmacophore by interligand nuclear Overhauser effect (ILOE), in which a pair of ligands that bind to adjacent pockets on the target surface is identified by the detection of protein-mediated ILOEs. We demonstrate that a pharmacophore-based search guided by experimental binding data of weakly interacting fragments can be rapidly and efficiently used to identify (or synthesize) high-affinity, selective ligands. Targeting the inactive state of protein kinases represents a promising approach to achieve selectivity and cellular efficacy. In this respect, when we apply the method for the discovery of potent p38α inhibitors, we also demonstrate that the resulting bidentate compounds are highly selective and exhibit a cellular activity that parallels their *in vitro* binding to the inactive form of the kinase. The method is relatively simple and of general applicability, and as such we anticipate its potential implementation against a variety of macromolecular targets, including not only protein kinases but also those involved in protein–protein interactions or even nucleic acids.

Mitogen-activated protein kinase (MAPK) p38 is responsible for the biosynthesis of inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) in lipopolysaccharide (LPS)-stimulated human monocytes (*1*). Among the four isoforms of p38, p38α is the best characterized and perhaps the most relevant for inflammatory responses, and it is therefore a promising drug target for various inflammatory diseases (*2, 3*).

One attractive avenue to design potent and selective protein kinase inhibitors is to rely on fragment-based approaches (*4–7*). A particular challenge in fragment-based drug discovery is the identification of initial binding fragments. Several biophysical methods, such as high-throughput X-ray crystallography, NMR, and mass spectrometry have been used to detect the binding of weak ligands to protein targets (*4–7*). To find new potent and selective p38α inhibitors, we adopted a novel lead discovery method, pharmacophore by interligand nuclear Overhauser effect (ILOE). This approach is based on the detection of protein-mediated ILOEs as a fragment-based screening method (*8–12*) (Figure 1). A pair of binders can be relatively easily identified from mixtures of fragments in a single experiment by detection of ILOEs (Figure 2, panel a). Such fragments are subsequently used to define a pharmacophore-based search for potential high-affinity bidentate compounds from commercial libraries (Figure 1). The ILOEs are also used to guide the synthesis of bidentate ligands from the individual fragments.

RESULTS AND DISCUSSION

In a typical 2D [^{1}H, ^{1}H] nuclear Overhauser effect spectroscopy (NOESY) experiment measured in the presence of a substoichiometric amount of protein, fragments

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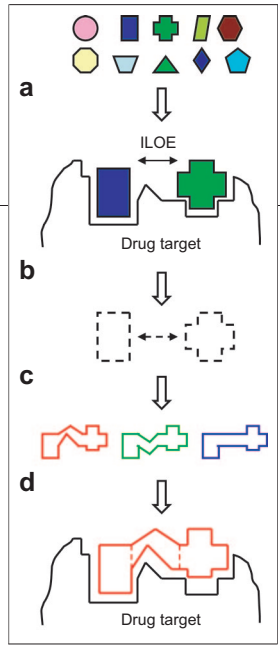


Figure 1. Schematic representation of the pharmacophore by ILOEs approach in the lead discovery process. a) A small but diverse fragment library is screened for binding to a drug target using ILOEs, whereby pairs of fragments that bind to adjacent pockets on the target surface are identified. b) The chemical substructures and relative orientation of the binding pair are used to define a pharmacophore. c) The pharmacophore is used to search for matches from commercially available libraries. d) The matches from the search are subsequently selected and tested.

that bind simultaneously in close proximity on the surface of the protein target are identified by detection of negative ILOE cross-peaks, which will have the same sign as the diagonal peaks (Figure 2, panel a). On the contrary, non-binding fragments would have either non-detectable or positive cross-peaks (Figure 2, panel a). Therefore, a pair of binders can be easily identified and distinguished from non-binders even when testing relatively complex mixtures of fragments. Here, we show the data relative to a single mixture containing 96 compounds from a fragment library in the presence of inactive p38α (Figure 2). Identification of the exact pair generating the ILOE cross-peaks can be obtained on the basis of the previously determined chemical shifts of each individual compound. Ambiguities can be resolved by further experimental testing to confirm the identity of the binding fragments (Figure 2, panel a). Subsequently, measurements of ILOE buildup rates with the identified pair can also provide important information regarding the relative orientation of the two fragments. Fragments **BI-14B5** and **BI-14D4** were found to have the strongest ILOEs that can be identified between the protons of the piperidine ring of **BI-14D4** and the phenyl ring of **BI-14B5** (Figure 2, panel b), suggesting that the two rings are in proximity (<5 Å) on the surface of p38α. Therefore, by using a mixture-based approach that relies on the detection of protein-mediated ILOEs, we discovered a pair of binding ligands and were able to roughly determine their relative orientation in the bound state. In order to critically assess whether the ILOEs are arising from ligand–ligand contacts and are not merely the result of protein-mediated spin-diffusion or compound aggregation, we performed several controls by measuring 2D NOESY of the mixture alone and in the presence of other proteins (Jun N-terminal kinase (JNK), B cell lymphoma (Bcl)-xL, heat shock cognate protein (Hsc) 70, Bcl-2 interacting domain (Bid), GST-baculoviral IAP repeat (BIR3)). We could not observe ILOEs on the mixture alone nor could we detect similar ILOEs in the presence of other proteins. Finally, we have also verified that ILOE cross-peaks between **BI-14B5** and **BI-14D4** are still present when using a perdeuterated sample of p38 (see Supplementary Figure 3), again largely attenuating any concerns about protein-mediated spin-diffusion (*13*). It is interesting to note that empirically we find that the probability of finding a pair varies from 1% (as for p38 shown here) to 5%, when the approach has been tested against the other above-mentioned proteins. Obviously, this hit rate could be further enhanced by testing, for example, focused or targeted libraries that are enriched in preferential scaffolds either by empirical dissection of known ligands or by computational approaches such as virtual docking. We also have found cases where the second binder is not detectable alone (by transferred nuclear Overhauser enhancement (trNOE)) when the first one is not present. Hence, in same cases we may conclude that the binding of the first ligand may help the formation of an extended binding site that is more receptive to second-site ligands.

Because fragments **BI-14B5** and **BI-14D4** bind to adjacent pockets on the surface of p38α, a pharmacophore was built based on the indole and *N*-phenylpiperidine rings, respectively, as illustrated in Figure 2, panel c. The atoms of both components were specified as "any atoms" to widen the pharmacophore-based search. No constraints were added between these two components. The program UNITY 2D search, as implemented in SYBYL, was used to search a commercial library of compounds, which resulted in a subset of 27 molecules that matched the pharmacophore. The chemical structures of the 27 compounds can be found as Supporting Information. Here, we want to emphasize that the chemical space covered by the single NMR experiment with 96 compounds is orders of magnitude larger than the mere 4560 pairs that one would calculate. Indeed, in the current example 27 molecules (and potentially many more considering various linker lengths and nature) are obviously represented by a single pair of fragments. Moreover, much as in other fragment-based strategies, fragment binders could be further individually optimized. Hence, the chemical space covered by a single NMR experiment is orders of magnitude larger that of any other approach where a single screening experiment is conducted. Obviously, the approach suits well the use of larger libraries and multiple NMR samples to increase even further the explored chemical space.

The ability of the 27 identified molecules to bind in the catalytic pocket of p38α was subsequently evaluated by a fluorescence assay based on the displacement of a known p38α inhibitor, SK&F 86002 (*14, 15*).

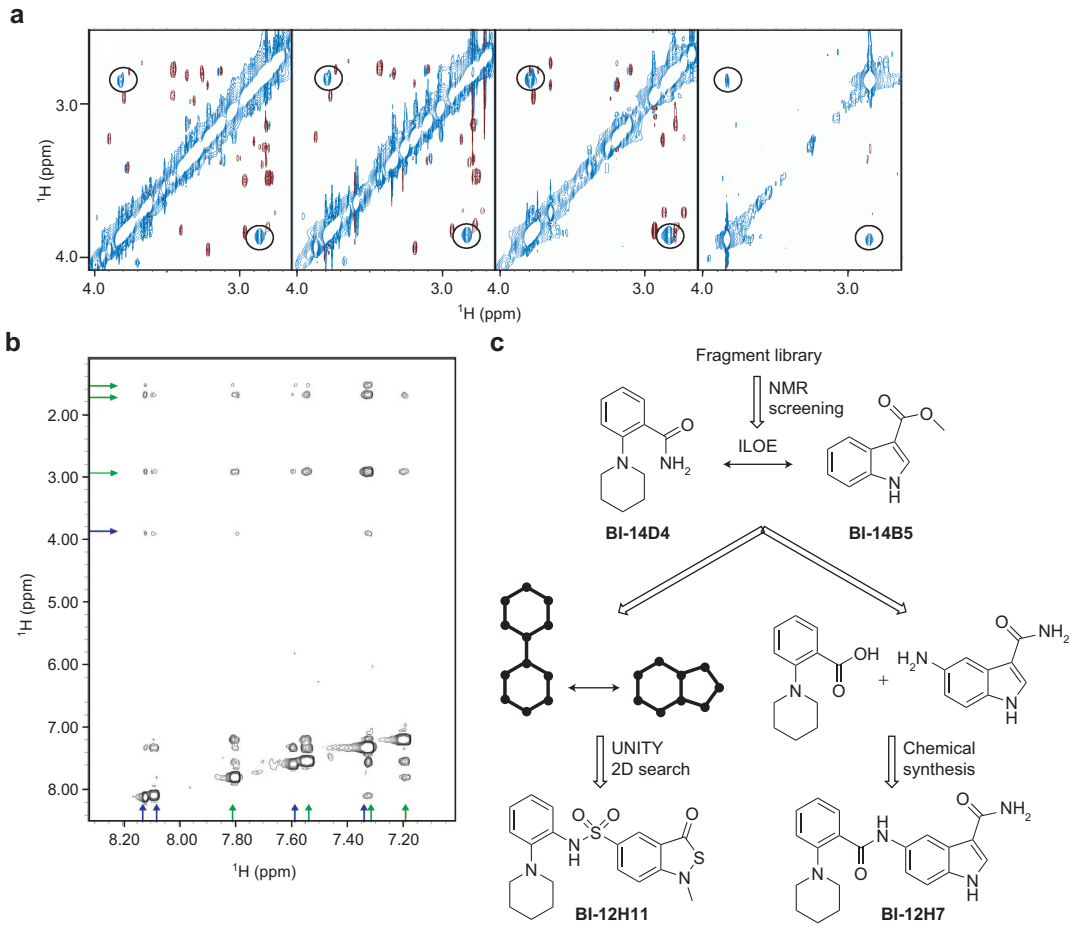


Figure 2. Pharmacophore by ILOEs as applied to p38α. a) A panel showing interligand NOEs in the aliphatic region of two binding fragments, BI-14B5 **(3.82 ppm for the α-methylene on the piperazine ring) and** BI-14D4 **(2.86 ppm for the methyl group) from the screening mixture. The total number of fragments in the mixture (from left to right) are 96, 48, 24 (200 μM each in presence of 5 μM p38α), and 2 (500 μM each in presence of 5 μM p38α), respectively. b) Intra- and interligand NOE cross-peaks between** BI-14B5 **and** BI-14D4 **(500 μM each) are detected in a 2D [^{1}H, ^{1}H] NOESY experiment with a mixing time of 600 ms, in the presence of a substoichiometric amount of p38α (5 μM). The NOE cross-peaks of** BI-14D4 **and** BI-14B5 **are labeled with green and blue arrows, respectively. c) Scheme for the discovery of p38α inhibitors from a pair of binding fragments by pharmacophore search and by chemical synthesis of a bidentate compound.**

Within this small library, we were able to identify a compound, **BI-12H11**, as a competitive ligand of SK&F 86002 for p38α (IC_{50} = 5.6 μM and K_i = 1.5 μM) (Figure 3, panel c). The binding of **BI-12H11** to p38α was further confirmed by isothermal titration calorimetry (ITC) (K_d = 0.76 μM) (Figure 3, panel a), in close agreement with the competitive displacement assay. Therefore, by using the pharmacophore by ILOEs approach, a potent p38α binder was discovered by testing a very small number of selected compounds based on ILOEs information.

Subsequently, the *in vitro* activity of **BI-12H11** was determined by the SelectScreen Z′-LYTE kinase profiling assay (Invitrogen, Carlsbad, CA). The IC_{50} value of **BI-12H11** for p38α was determined to be 709 nM (Figure 3, panel c). The selectivity of **BI-12H11** for p38α was also tested (Table 1). When tested at 10 μM, **BI-12H11** showed total inhibition against p38α, as compared to

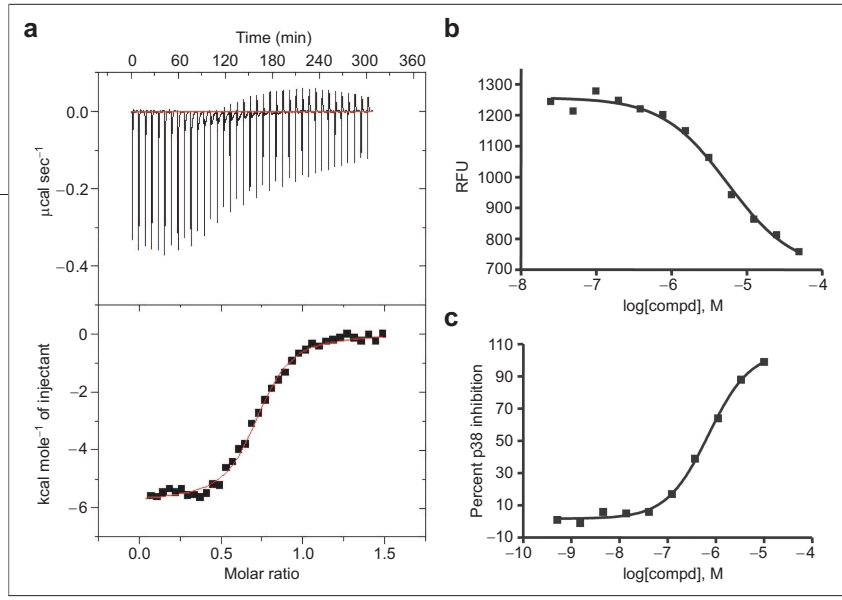


Figure 3. Biological evaluation of BI-12H11**. a) The dissociation constant value (K_d) for p38α was 0.76 μM as determined by ITC. b) The IC_{50} value was 5.61 μM (K_i = 1.5 μM) as determined by a fluorescence-based competition assay with SK&F 86002 to p38α. c) The kinase inhibitory activity IC_{50} value of** BI-12H11 **for p38α was 0.71 μM as determined by the Invitrogen SelectScreen Z′-LYTE assay.**

only 28% inhibition against p38β and no significant inhibition against p38γ and p38δ. Moreover, closely related MAPKs, such as extracellular signal-regulated kinase (ERK) 1 and ERK2 (data not shown) showed <30% inhibition at 10 μM of **BI-12H11**. MAPK and ERK kinase 1 (MEK1) was also inhibited by **BI-12H11** at 10 μM (65%). The IC_{50} value of **BI-12H11** against MEK1 was further determined to be 4.2 μM. Therefore, the selectivity of **BI-12H11** for p38α over the most appreciably inhibited kinases on the panel was ~6-fold. However, the most notable feature of the inhibitory activity of **BI-12H11** is its unique ability to selectively target the α isoform of p38 (Table 1). Compared to various well-characterized p38α inhibitors, such as SB202190 (*2*) and BIRB796 (*14, 15*), our compound showed a remarkable isoform specificity that was not observed in the inhibitors reported thus far (Table 1).

TABLE 1. Profiling kinase selectivity of BI-12H11 and previously reported p38 inhibitors BIRB796 and SB202190

Kinase	ATP concentration (μM)	% Inhibition at 10 μM		
		BI-12H11	BIRB796	SB202190
MAPK14 (p38α)	100	102	99	103
MAPK11 (p38β)	$K_{m\ app}$	28	94	96
MAPK12 (p38γ)	$K_{m\ app}$	7	96	12
MAPK13 (p38δ)	$K_{m\ app}$	0	76	7
EGFR (ErbB1)	$K_{m\ app}$	0	59	74
LCK	$K_{m\ app}$	0	49	37
MAP2K1 (MEK1)	100	65	2	14
MAPK3 (ERK1)	$K_{m\ app}$	30	2	1
PRKACA (PKA)	$K_{m\ app}$	11	2	64
PRKCA (PKCα)	$K_{m\ app}$	34	8	5

Inhibition of p38α and subsequent modulation of TNF-α and IL-1 is an attractive approach for the treatment of inflammatory diseases (*1*). Therefore, the effect of **BI-12H11** on TNF-α production in THP-1 cells induced by *Escherichia coli* LPS was determined. After cells were treated with compounds at different concentrations, TNF-α production was induced by LPS and measured directly from the cell culture medium by a commercially available sandwich immunoassay (Meso Scale Discovery). We found that when the cells were treated with **BI-12H11**, production of TNF-α was significantly reduced in a dose-responsive manner (Figure 4). Fragments **BI-14B5** and **BI-14D4** did not show appreciable binding to p38α from either the fluorescence displacement assay or the ITC experiment (up to 200 μM), indicating that the binding affinity of these fragments for p38α is in the millimolar range. Accordingly, **BI-14B5** and **BI-14D4** did not show measurable inhibition of TNF-α production up to 100 μM (Figure 4).

Recently, we reported a fragment-based lead discovery method called structure–activity relationship (SAR) by ILOEs (*9–12*), in which novel high-affinity ligands are discovered by covalently linking fragments of weak affinity as guided by ILOEs. Inspired by the structure of **BI-12H11** and by ILOEs data (Figure 2), we designed and synthesized the bidentate compound **BI-12H7** by covalently linking easily accessible building blocks similar to **BI-14B5** and **BI-14D4** (Scheme 1). **BI-12H7** showed appreciable binding to p38α as estimated by ITC (K_d ~20 μM) but limited activity against p38α in an *in vitro* kinase assay (11% at 25 μM). Therefore, **BI-12H7** binds to the inactive form of p38α (as per ITC data) better than to its activated form (*in vitro* kinase assay). This might be due to the fact that the original binding fragments **BI-14B5** and **BI-14D4** were discovered by screening against the inactive form of p38α. In the cell-based assay, however, **BI-12H7** showed significant inhibition of TNF-α production (Figure 4). Hence, targeting the inactive form of the kinase resulted in more efficacious and selective inhibitors (*16*).

High-throughput screening (HTS) has been widely used in the lead discovery process. A successful HTS

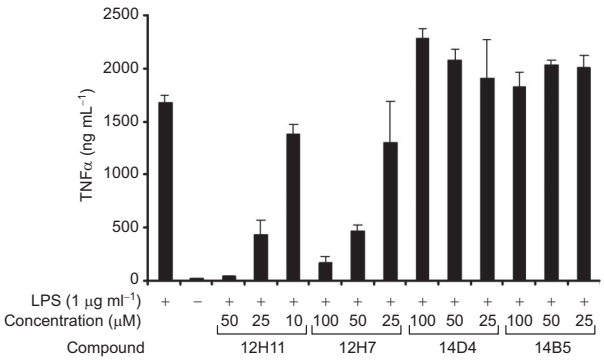
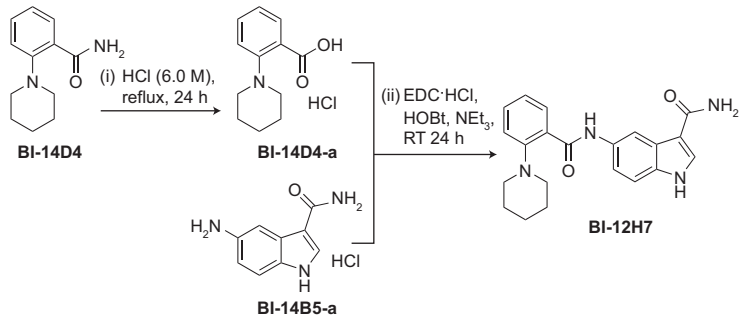


Figure 4. TNF-α production inhibition by the bidentate compounds in cell-based assays. THP-1 cells were preincubated with controls and compounds, added at different concentrations, prior to stimulation with *E. coli* LPS. TNF-α production was measured directly from cell culture medium by a sandwich immunoassay (Meso Scale Discovery).

campaign usually requires a robust assay. However, not all assays can be easily and economically implemented in an HTS format (*17, 18*). The quality and diversity of the screening compound libraries are also very critical for the success of an HTS campaign. Unfortunately, many commercially available libraries may contain compounds with undesirable properties. For example, parallel to our fragment-based discovery, we have also screened a commercial library of 14,000 compounds, using the fluorescence displacement assay described earlier. We find that all of the top hits are "frequent hitters" (*19*) and promiscuous aggregators (*20, 21*) (see the Supplementary Table for the chemical structures of these hits). Hence, by exploring a chemical space that is much larger in size, the fragment-based approach provided compounds that are superior in terms of selectivity and drug-likeness to those obtainable by a typical HTS approach.

Virtual screening has also been used successfully in improving hit rates in many drug discovery programs (*22*). However, usually an initial high-quality structure of a ligand–protein complex is needed for optimal results. Fragment-based drug discovery has recently emerged as a complementary approach to these endeavors (*23, 24*). Only a relatively small fragment library is needed to sample a relatively large chemical space (*25*), and because only compounds derived from binding fragments are tested, only a small number of compounds are screened. Compared to other fragment-based discovery methods, the pharmacophore by ILOEs approach is relatively easily implemented. Successful fragment-based approaches include high-throughput X-ray crystallography (*4*), and the SAR by NMR approach (*26*), both relying heavily on determination of the high-resolution structures of the ternary complexes. The pharmacophore by ILOEs approach is complementary and likely more efficient than these techniques, as it bypasses the structure determination process and even the synthesis of the compounds altogether.

In practical terms, the pharmacophore by ILOEs method requires the collection of only a few dozens of 2D [^{1}H, ^{1}H] NOESY experiments for the screening a fragment library. This screen allows the sampling of a chemical space that is orders of magnitudes larger than what is covered by typical HTS libraries, given all the possible combinations that pairs of fragments can adopt on the surface of the target. A relatively small amount of protein is needed for the screening (0.5 mL, 1–10 μM protein for each sample for a typical NOESY experiment) when compared to other fragment-based approaches. Since it is the fragment that is observed in the NOESY experiment, the protein sample does not need to be isotopically labeled and there is no upper limit to the size of the target. Because the detection of the binding fragments is not based on a functional assay, this method could also be applied to the discovery of small molecules that modulate protein–protein interactions (*23*) or, as shown here, to target protein kinases in their inactive state. As we demonstrated in the example reported here, an inhibitor targeting the kinase at its inactive state tends to have better cellular activity and selectivity (*16*).



Scheme 1. Synthesis of bidentate compound BI-12H7**.**

Conclusion. The pharmacophore by ILOEs method offers a valuable alternative to current lead discovery methods, such as HTS, virtual screening, and even other fragment-based methods. Compared to other methods, the pharmacophore by ILOEs is less resource-intensive, less time-intensive, and readily available. It is complementary to other methods and especially useful at the early stage of the hit discovery program where there is no structural information of the target and when HTS assays are not easily implemented.

METHODS

Design of a Diverse Fragment Library. Fragment libraries have been commonly assembled on the basis of structural diversity, drug scaffolds, or target-oriented privileged compounds by using either computational or empirical methods (*5, 6, 23*). Our fragment library contains ~490 diverse compounds selected from the MayBridge collection. These fragments were first selected on the basis of structure diversity and the "Rule of Three" (molecular weight < 300, cLogP < 3, hydrogen bonding donor and acceptor < 3) (*28*). Fragments that contain reactive groups such as aldehyde, Michael acceptor, epoxide, and aziridines were not included. These fragments were further selected on the basis of their commercial availability and cost for eventual follow-up synthesis. The chosen fragments were dissolved in DMSO-d_6 at 200 mM, and the ^{1}H NMR spectrum of each fragment was acquired in pD 7.5, 50 mM phosphate buffer in D_2O, as standard for identification of the fragment in mixture. For the screening purpose, 2–96 fragments were mixed in a final concentration varying from 200 μM (in larger mixtures) to 500 μM (when testing the two final compounds) each in the presence of 5 μM p38α. In this particular example, only 96 fragments were tested in a single NMR experiment, and a pair was identified by subsequent deconvolutions as shown in Figure 2, panel a.

Protein Expression and Purification. Recombinant human inactive MAPK p38α with 23 extra amino acid residues (MGSSHHHHHHSSGLVPRGSHMAS) at the N-terminus was produced from a pET28a plasmid (Novagen) in *E. coli* $BL_{21}(DE_3)$ in LB media at 25 °C with an overnight induction of 1 mM IPTG. The protein was subsequently purified over a Hi-Trap Ni^{2+} affinity column (Amersham, Pharmacia). Similarly, U-^{2}H p38 was obtained by bacterial growth in M9 minimal medium in 90% ^2H_2O (CIL).

NMR Spectroscopy. All NMR experiments were measured in pD 7.5, 50 mM phosphate buffer at 25 °C. The experiments were performed on 600 MHz Bruker Avance spectrometers, both equipped with a TXI cryoprobe and an automatic sample changer. 2D [^{1}H, ^{1}H] NOESY spectra were typically acquired with 32–128 scans for each of 128 indirect points, a sweep width of 12 ppm in both directions, a mixing time of 510–800 ms, and a recycle delay time of 1 s. In all experiments, residual water signal dephasing was obtained by a WATERGATE sequence.

Pharmacophore-Based Search. All computational studies were carried out on a Linux workstation. The pharmacophore was built based on the *N*-phenylpiperidine and indole ring of **BI-14D4** and **BI-14B5**, respectively. The atoms of both components were specified as "any atoms" to widen the subsequent pharmacophore-based search. No constraints were added between these two components. UNITY 2D search as implemented in SYBYL version 7.2.3 (TRIPOS, St. Louis, MO) was carried out to search for compounds that satisfy such a pharmacophore from a library of 30,000 compounds (*29*). The screening generated 27 matches. Currently, structural information of millions of commercially available compounds have been compiled to central databases, such as ZINC (*30*) and ChemNavigator (ChemNavigator.com). These databases could be similarly easily used for pharmacophore-based searches as guided by ILOEs.

General Synthetic Procedures. All commercially available reagents were used without further purification. Column chromatography was performed with silica gel 60 (35–75 Å). ^{1}H NMR and ^{13}C NMR spectra were acquired on a Varian Inova 300 MHz spectrometer. Chemical shifts are reported in ppm from residual solvent peaks (2.50 and 3.31 for DMSO-d_6 and CD_3OD, respectively, for ^{1}H NMR; 49.0 and 39.5 for DMSO-d_6 and CD_3OD, respectively, for ^{13}C NMR). High resolution electrospray ionization-TOF mass spectra were acquired at the Center for Mass Spectrometry, the Scripps Research Institute, La Jolla, CA.

2-(Piperidin-1-yl)benzoic Acid (BI-14D4-a). 2-(Piperidin-1yl)benzamide (**BI-14D4**) (408.54 mg, 2.0 mmol) was added to an aqueous HCl solution (6.0 M, 10 mL) and heated under reflux under nitrogen for 24 h. The solvent was removed under reduced pressure, and the residue was dissolved in methanol (MeOH, 15 mL). After the solution was concentrated (5 mL), ether (20 mL) was added, and the mixture was kept at 0 °C for 1 h. The white precipitate was collected by filtration and washed with cold ether to give **BI-14D4-a** as a chloride salt (300 mg, 62%). ^{1}H NMR (300 MHz, CD_3OD) δ 8.32–8.28 (m, 1 H), 8.09 (d, $J = 8.4$ Hz, 1 H), 7.96–7.90 (m, 1 H), 7.75–7.70 (m, 1 H), 3.76 (s, 4 H), 2.11 (s, 4 H), 1.90 (s, 2 H). ^{13}C NMR (75 MHz, CD_3OD) δ 170.8, 144.9, 136.9, 133.5, 131.1, 123.2, 122.5, 58.0, 26.1, 22.2. HRMS *m/z* calcd for $C_{12}H_{16}NO_2$ $[M + H]^+$ 206.1175, found 206.1179.

5-(2-(Piperidin-1-yl) benzamido)-1 *H*-Indole-3-carboxamide (BI-12H7). *N*-Ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl, 229.2 mg, 1.2 mmol), 1-hydroxy-1*H*-benzotriazole hydrate (HOBt, 183.6 mg, 1.2 mmol), and triethylamine (400 mg, 4.0 mmol) were added to a solution of **BI-14D4-a** (242 mg, 1.0 mmol) in dimethylformamide (DMF) (10 mL). After the solution was stirred at RT for 20 min, 5-amino-1 *H*-indole-3-carboxamide (**BI-14B5-a**) (210 mg, 1.0 mmol) was added and stirred at RT for 24 h. DMF was removed under reduced pressure, and the residue was partitioned between ethyl acetate (EtOAc, 50 mL) and water (30 mL). The EtOAc solution was washed with water (3×20 mL), concentrated (5.0 mL), and kept at 0 °C. The precipitate was collected by filtration and washed with cold EtOAc to give **BI-12H7** as an off-white solid (20 mg, 6%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.94 (s, 1 H), 11.51 (s, 1 H), 8.60 (d, $J = 2.1$ Hz, 1 H), 8.02 (d, $J = 2.7$ Hz, 1 H), 7.95 (dd, $J = 1.8$ Hz, 7.8 Hz, 1 H), 7.58–7.49 (m, 2 H), 7.43–7.36 (m, 2 H), 7.28–7.21 (m, 1 H), 3.02–2.94 (m, 4 H), 1.80–1.69 (m, 4 H), 1.61–1.52 (m, 2 H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 166.5, 163.7, 152.0, 133.0, 132.4, 131.9, 130.3, 128.9, 128.6, 126.6, 124.0, 120.9, 115.6, 112.0, 111.8, 110.4, 54.0, 26.0, 23.3. HRMS *m/z* calcd for $C_{21}H_{23}N_4O_2$ $[M + H]^+$ 363.1815, found 363.1819.

Fluorescence-Based Displacement Assay. The binding affinity of ligands to p38α was determined using a simple fluorescence-based assay (*14, 15*). The assay was based on the competitive binding between the testing compounds and a known fluorescent p38α inhibitor, SK&F 86002 ($K_d = 180$ nM). After test compounds were diluted into the binding buffer (100 μL, pH 7.0, 20 mM Bis-Tris propane, containing 0.15% *n*-octylglucoside and 2.0 mM EDTA) in a 96-well plate, the eventual background fluo-

rescence of each compound was recorded at an excitation of 340 nm and emission of 420 nm. A 100-μL mixture of p38α (1.0 μM) and SK&F 86002 (1.0 μM) was then added to each well. After the plate was incubated at RT for 60 min, the fluorescence was recorded, and the background fluorescence was subtracted. A known inhibitor, SB203580, was used as control. The binding constant was calculated by directly fitting the fluorescence value using one site competitive mode in Prism (Graphpad software, San Diego, CA).

***In Vitro* Kinase Activity Assay.** The activity and selectivity of the kinase inhibitors were determined by SelectScreen kinase profiling (Invitrogen, Carlsbad, CA). All kinases, except MEK1 and p38α, were initially tested at an inhibitor concentration of 10 μM, and the ATP concentration was set at the respective K_m values, using a direct Z′-LYTE biochemical assay. For MEK1 and p38α, the inhibition of the kinase was determined using a cascade format at 10 μM of compound and 100 μM of ATP (Table 1). For MEK1 assay, MEK1 was used to activate ERK2, and the activity of ERK2 was determined. For the p38α assay, p38α was used to activate MAPKAP-K2 (MK2) and the activity of MK2 was determined. The inhibition of the downstream kinases, ERK2 and MK2, was also determined as control. **BI-12H11** showed some inhibition to MK2 (IC_{50} = 9.33 μM) but only contributed insignificantly to the inhibition of p38α (IC_{50} = 0.709 μM).

ITC. The ITC experiments were performed using a Microcal VP-ITC calorimeter (Northampton, MA). The sample cell of the calorimeter was filled with a solution containing inactive p38α (100 μM) in 50 mM phosphate buffer (pH 7.5) and 10% DMSO. The syringe was loaded with the testing compound in the same buffer with 10% DMSO.

THP-1 Cell Assay for Inhibition of LPS-Induced TNF-α Production. THP-1 cells (ATCC TIB 202, ATCC, Rockville, MD) were maintained at 37 °C, 5% CO_2 in 10% fetal bovine serum (FBS)/RPMI 1640 medium. The day of the assay, 2×10^6 cells were resuspended in 1 mL of 3% FBS/RPMI 1640 medium and plated in a 12-well plate. Test compound or DMSO vehicle was added to the cell mixture and allowed to preincubate for 40 min at 37 °C, 5% CO_2, prior to stimulation with LPS (Sigma L6529, from *E. coli* serotype 055:B5; 1 μg/mL final). LPS stimulation was allowed to proceed for 5 h at 37 °C, 5% CO_2. TNF-α production was measured directly from cell culture medium by a commercially available sandwich immunoassay developed by Meso Scale Discovery (Meso Scale no. K151BHB-1, Gaithersburg, MD). Levels of TNF-α in the cell culture medium were determined using a Meso Scale Discovery Sector Imager 2400 according to the manufacturer's instructions. We have also verified that the inhibition of the TNF-α production is not simply a general toxic effect of the compounds as determined by their effect on the β-actin levels (data not shown).

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Supporting Information Available: This material is available free of charge *via* the Internet.

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