Bioorganic & Medicinal Chemistry Letters 18 (2008) 4433–4437

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Biphenyl amide p38 kinase inhibitors 4: DFG-in and DFG-out binding modes

Richard M. Angell †, Tony D. Angell, Paul Bamborough *, Mark J. Bamford, Chun-wa Chung, Stuart G. Cockerill †, Stephen S. Flack †, Katherine L. Jones, Dramane I. Laine, Timothy Longstaff, Steve Ludbrook, Rosannah Pearson, Kathryn J. Smith, Penny A. Smee, Don O. Somers, Ann L. Walker

GlaxoSmithKline R&D, Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY, UK

ARTICLE INFO

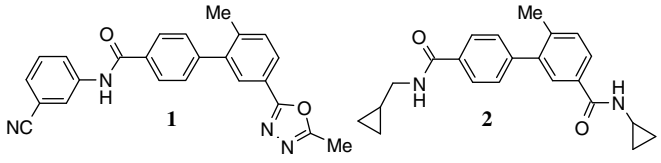
Article history:
Received 9 May 2008
Revised 6 June 2008
Accepted 6 June 2008
Available online 12 June 2008

Keywords:
p38 Kinase inhibitors
MAP kinase
Biphenyl amide
Protein kinase X-ray structure
Binding mode
DFG-out
Selectivity
Kinetics

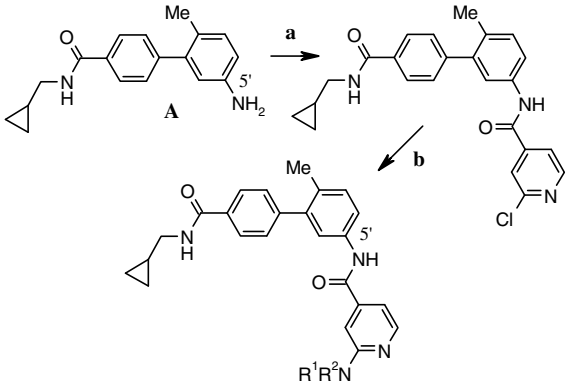
ABSTRACT

The biphenyl amides (BPAs) are a series of p38α MAP kinase inhibitors. Compounds are able to bind to the kinase in either the DFG-in or DFG-out conformation, depending on substituents. X-ray, binding, kinetic and cellular data are shown, providing the most detailed comparison to date between potent compounds from the same chemical series that bind to different p38α conformations. DFG-out-binding compounds could be made more potent than DFG-in-binding compounds by increasing their size. Unexpectedly, compounds that bound to the DGF-out conformation showed diminished selectivity. The kinetics of binding to the isolated enzyme and the effects of compounds on cells were largely unaffected by the kinase conformation bound.

The biphenyl amides (BPAs) are a novel series of p38α MAP kinase inhibitor.[1] Replacing the oxadiazole moiety found in the earliest examples (as in **1**) with amides led to compounds with improved enzyme, cellular and in vivo activity, for example, **2**.[2,3] An X-ray structure of p38α complexed with **2** showed binding to the *apo*-like (or 'DFG-in') conformation.[3]



Prior to the publication of the X-ray structure of p38α with BIRB-796, and of Abl with Gleevec, compounds had been prepared within GSK which revealed the possibility of binding to a rearranged ('DFG-out') form of p38α.[4–11] Large substituents were attached to the BPA template with the aim of exploiting the DFG-out pocket. Initial work focused on anilides (**4**–**15**), prepared from the 5′-aniline position (Scheme 1).



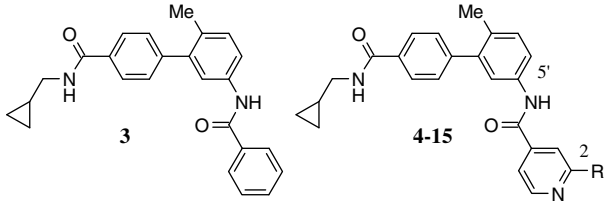
Scheme 1. Intermediate **A** was prepared as previously described.[3] Reagents: (a) 2-chloropyridine-4-carbonyl chloride, NEt_3, DCM, 33%. (b) Amine, heat.

The phenyl-substituted anilide **3**, with p38α K_i of 12 nM, provides a benchmark for the activity of **4–15** (Table 1).[3] 4-Amido pyridines with small 2-substituents such as **4** were weakly active. Activity was increased by the introduction of moderate bulk at the 2-position (**5**). Many compounds with increased bulk at this position, particularly aliphatic rings, gave greater activity (**6–14**), especially the cyclobutyl amide **13**. Cationic charge in this region was not tolerated (compare **15** to **9**).

* Corresponding author. Tel.: +44 1438 763246; fax: +44 1438 763352.
E-mail address: paul.a.bamborough@gsk.com (P. Bamborough).
† Present address: Arrow Pharmaceuticals, Britannia House, 7 Trinity Street, London SE1 1DB, UK.

0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.06.028

Table 1
K_i (nM), M_W and BEI of pyridinyl anilides[12]



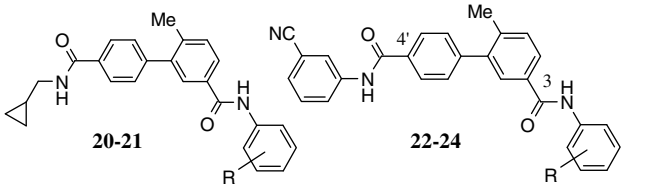
Compound	R	K_i	M_W	BEI
1[2]	n/a	240	394	17
2[3]	n/a	12	348	23
3[3]	n/a	12	384	21
4	Cl	150	420	16
5	NMe_2	14	429	18
6	1-Pyrrolidine	1.9	455	19
7	1-Piperidine	2.5	469	18
8	1-Morpholine	4.4	471	18
9	(4-Methyl)-1-piperidine	15	483	16
10	NH-cyclopropyl	5.2	441	19
11	NH-methylcyclopropyl	6.3	455	18
12	NH-isobutyl	4.6	457	18
13	NH-cyclobutyl	0.6	455	20
14	NH-cyclohexyl	3.0	483	18
15	(4*N*-methyl)-1-piperazine	300	484	13

Substituted phenyls showed similar trends to the pyridines (Table 2).[13] Compared to the unsubstituted phenyl **3**, and the furan **16**, even small 4-substituents led to reduced activity (**17**). Acids chosen to introduce bulky groups *meta* to the amide (e.g., **18**, **19**) increased the activity.

The same trends were seen in the benzamide series (Table 3).[13] Although the phenyl **20** was less potent than the corresponding anilide **3**, introduction of bulky *meta* substituents (**21**) gave the same increase in activity.

The 4′-amide group could be varied using previously described chemistry.[13] The cyanophenyl was known to give good potency in DFG-in binding BPAs.[3] With the same 4′-group, **22** showed good activity (Table 3). The relatively poor inhibition of the 3-acetamide analogue (**23**) illustrates again the importance of a *meta* group with sufficient size and lipophilicity. As in the anilide series, *para* substitution (**24**) reduced the potency.

Table 3
K_i (nM), M_W and BEI of aryl benzamides[12]

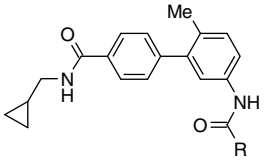


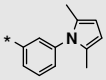
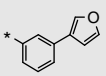
Compound	R	K_i	M_W	BEI
20	H	73	384	19
21	3-^{t}Bu	1.9	441	20
22	3-(2-Pyridyl)	11	509	16
23	3-(NHCOMe)	79	489	15
24	4-OMe	730	462	13

The crystal structure of p38α complexed with **8** was solved and found to adopt a rearranged DFG-out conformation.[14] The backbone around the DFG motif reorganises in conjunction with the activation loop. The Phe169 sidechain moves some 12 Å relative to the complex with **2**, to a new location around the ATP-site sugar pocket. Figure 1 shows the binding site in the region of the DFG-out pocket. The morpholine ring of **8** fills the lipophilic space that is occupied by Phe169 in the complex with compound **2** (the DFG-pocket). It makes direct contact with the sidechains of residues including Leu74, Met78, Val83, Ile141 and Ile166. The motion of Phe169 out of this pocket is accompanied by a small inward movement of Met78 towards the inhibitor morpholine. Figure 1 also shows the details of the hydrogen-bonding interactions between the protein and 5′-amide of **8**. Two hydrogen-bonds are conserved in **2** and **8**, between the backbone NH of Asp168 and the amide carbonyl, and between the Glu71 sidechain and the amide NH.

The interactions of the hinge-binding parts of **8** and **2** with the protein are similar, but the biphenyl amides of **8** and **2** are shifted relative to one another (Fig. 2). The backbone around Met109 and Gly110 in the complex with **8** has some features of the *apo*-like conformation seen with **2** and some of the flipped conformation seen with **1**.[1,3] In complex with **8**, the carbonyl of Met109 points towards the ATP-site as in the *apo* structure. However, the overall shape of the hinge is more like that seen with **1** than that with **2**. The movement of the hinge-binding amide of **8** relative to **2** prob-

Table 2
K_i (nM), M_W and BEI of aryl anilides[12]



Compound	R	K_i	M_W	BEI
3[3]	Phenyl	12	384	21
16[3]	3-Furan	15	374	21
17	4-Methyl phenyl	100	399	18
18		2.1	478	18
19		1.6	451	20

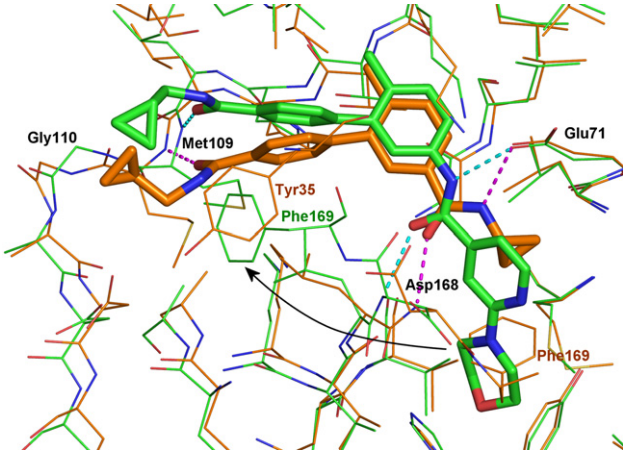


Figure 1. Overlaid p38α X-ray structures of **8** (green) and **2** (orange), focusing on the part of the inhibitors around the DFG-pocket. H-bonds are shown as dotted lines, in cyan from **8** and magenta from **2**. The arrow shows the movement of Phe169.

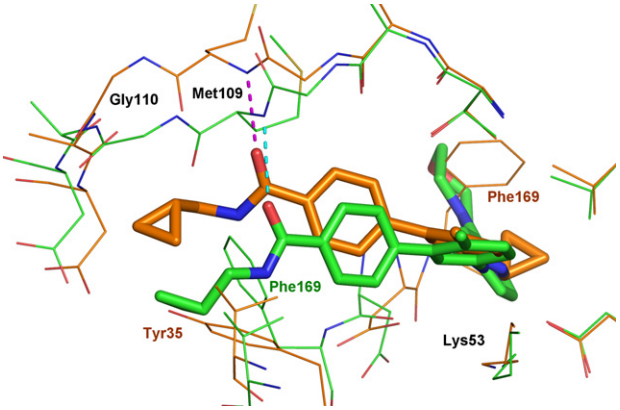


Figure 2. Overlaid X-ray structures of **8** (green) and **2** (orange), focusing on the part of the inhibitors around the hinge region. H-bonds to the hinge are shown as dotted lines.

ably results from the DFG-out position of Phe169, which would clash with **2** in the overlaid structures.

The binding modes of BIRB-796 and **8** are shown in Figure 3. The binding interactions of **8** are comparable to this and other published DFG-out crystal structures.[4] The amide of **8** makes the same interactions to Asp168 and Glu71 as the urea of BIRB-796, and the morpholine occupies the same pocket as the *tert*-butyl group of BIRB-796. The SAR in Tables 1–3 can be explained as follows. Groups with small amide substituents (like **2**, **3** and **16**) are highly potent and bind to p38α in the DFG-in conformation. Compounds with large *meta*-substituted aryl amides (e.g. **8**) would clash with Phe169 in this conformation. Instead, they take advantage of the alternative DFG-out conformation. Compounds such as **4** are of intermediate size and are weaker inhibitors than smaller or larger ones. In the DFG-in conformation, they are large enough to clash with Phe169, but not large enough to fill the pocket vacated by Phe169 in the DFG-out conformation. One interpretation is that adopting the DFG-out conformation incurs an energetic penalty which can only be overcome by making significant lipophilic interactions with the Phe-out pocket.

As well as being competitive with the fluorescent ATP-site ligand used to generate the SAR reported here, compounds **2** and **8** were both competitive with ATP in an assay measuring the catalytic activity of activated p38α, with K_i of 9 and 6 nM, respectively.[15] In the same assay, the K_i of BIRB-796 decreased 2.5-fold from 46 to 19 nM after 90 min of compound preincubation, indicating a slow on-rate, consistent with the literature.[4,8] Compound **8** showed no time-dependence under the same conditions.

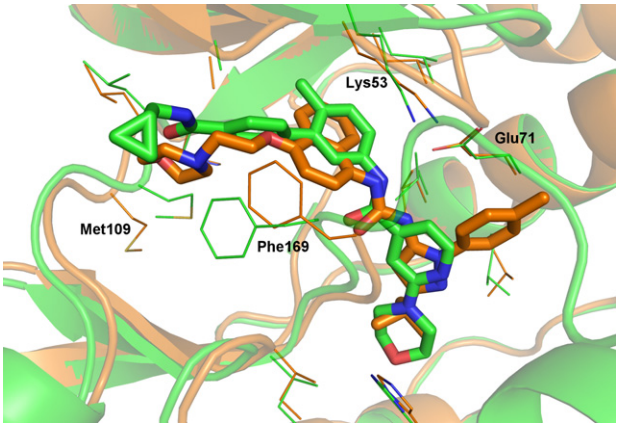


Figure 3. p38α X-ray structures of **8** (green) and BIRB-796 (orange).

It is difficult to obtain accurate K_i values for compounds with very slow kinetics in the catalytic activity assay. Comparative direct binding data for **2**, **8** and BIRB-796 to immobilised unphosphorylated p38α were obtained using surface plasmon resonance.[16] Both on- and off-rates of **8** are slightly slower than those of **2**, but both are much faster than BIRB-796 (Fig. 4). The rate constants (Table 4) are comparable to published values for the literature compounds.[7,8,17]

It has been suggested that the slow association is rate-limited by structural reorganisation of p38α from DFG-in to DFG-out, influenced by features of the inhibitor that hinder access to the bioactive conformation.[6,7] However, despite the similarity of the protein structures in complex with **8** and BIRB-796, **8** shows a much more rapid on-rate. This suggests that there may be a barrier due to conformational changes in the protein, but this is relatively minor compared to the effects of the characteristics of the inhibitor. One possible explanation is that the ATP-site part of **8** is less flexible, and so may spend more time in its bioactive conformation.

Whilst a slow on-rate is unlikely to be beneficial, a slow off-rate may be. BIRB-796 has a very slow k_{off} (Fig. 4). The off-rate of BIRB-796 depends largely on the interactions between protein and ligand in the bound state.[7] For example, replacing the tolyl ring of BIRB-796 with methyl led to a 400-fold increase in k_{off}. This is consistent with our results. **8** binds in the DFG-out mode but does not fill the tolyl space (Fig. 3) and has a faster k_{off}. More extensive interactions with the site might decrease the dissociation rate. Given the lipophilic nature of the p38α active site, this is likely to require compounds with increased molecular weight.

In consequence, DFG-out BPA compounds can reach higher potency than DFG-in compounds, but they are larger molecules, with M_W ~500, regarded as disadvantageous for oral drugs.[18] Molecular weight and BEI, a measure of binding efficiency, are shown in Tables 1–3 and 5.[12] DFG-out compounds do not gain enough activity from their extra mass to give them the efficiency of potent DFG-in compounds (compare compound **2** to **13**).

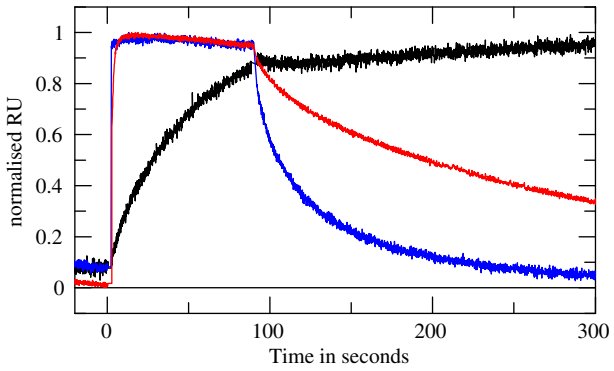


Figure 4. Normalised sensorgrams of compounds **2** (blue), **8** (red) and BIRB-796 (black) at 3.7 μM.[16] Compounds were injected at 0 s. The traces from 0 to 90 s show the association phase. Compound **2** shows the most rapid on-rate, closely followed by **8**, whilst BIRB-796 barely reached equilibrium during the 90 s. The traces from 90 to 300 s show the dissociation phase of these compounds and a similar hierarchy in off-rates, **2** > **8** ≫ BIRB-796.

Table 4
On- and off-rates from SPR measurements[16]

Compound	DFG	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	k_{off}/k_{on} (nM)
SB-203580	In	1.1×10^6	0.016	15
2	In	5.8×10^6	0.023	4
8	Out	1.8×10^6	0.0084	5
BIRB-796	Out	3.5×10^4	<0.00001	<0.5

All reported crystal structures of p38α have used inactive protein, unphosphorylated on T180 and Y182. The DFG-out conformation appears to be incompatible with phosphate transfer, and so has been described as an 'inactive conformation'.[19] Nevertheless, BIRB-796 binds to phosphorylated and unphosphorylated p38α, suggesting that both states of the kinase sample the DFG-out conformation.[8] The ability of compounds to bind to p38α and block its activation has been reported in vitro and in cells.[8,20–23] BIRB-796 has shown this activity in all studies. One interpretation is that DFG-out compounds can lock p38α into a state that cannot be activated, giving them a potential advantage. However, results for DFG-in compounds in the same studies have been inconsistent.

To investigate this, two DFG-in and two DFG-out BPAs were tested in IL-1-stimulated HLF cells for their ability to inhibit phosphorylation of a downstream marker of the p38 pathway, Hsp27, and of p38α itself.[24] All compounds showed potent inhibition of both readouts, as did two standards, BIRB-796 and SB-242235 (Table 5).[25] The ratio of the two readouts should normalise for compounds with different cell permeability, and is comparable for all compounds irrespective of their binding conformation. If anything, for reasons that are unclear, the BPAs inhibit p38α phosphorylation to a greater extent than Hsp27 phosphorylation. The effect on p38α phosphorylation differs from some published data, perhaps because of different cell types, stimuli or incubation times, but others have also found that DFG-in compounds can block p38α phosphorylation.[21,23]

K_i values for binding to the p38α enzyme are compared to IC_{50} for inhibition of TNF-α production in PBMC cells in Table 5.[12] The ratio between enzyme and PBMC activities is comparable for all four BPAs. Binding to different enzyme conformations does not affect TNF-α production in LPS-stimulated PBMC cells over the timescales used in the assay (18–20 h). It is unclear why lower ratios are seen for BIRB-796 and SB-242235. These clinical candidates may simply have better cell permeability, but other causes may be slow kinetics in the case of BIRB-796 or inhibition of off-target kinases.

The selectivity profiles of exemplar DFG-in and DFG-out BPAs are summarised in Table 6. Out of 35 protein kinases tested, **7** inhibited cRaf (80 nM), Lyn (4 μM) and murine Lck (5 μM). It had IC_{50} > 10 μM against the others, including JNKs 1 and 3. In addition, compounds were screened in the KinomeScan binding assay panel of 203 kinases.[26] **7** displaced ATP-site ligands from several kinases (Table 6). Some of these overlap with published activities of BIRB-796 against a smaller panel of 116 kinases, which did not include LOK or RAF1. BIRB-796 reportedly inhibited STK10, the JNKs, p38γ, SLK, TEK and TNIK with sub-micromolar K_d.[26] Whilst significant activity against Lck and JNK was not seen with **7**, some other DFG-out BPAs did show these activities. This complex profile contrasts with that of the selective DFG-in p38α/β inhibitor **2**, which has shown no significant inhibition of any other kinase.[3]

It is sometimes stated that binding to a DFG-out kinase conformation can be used to gain improved selectivity. This assertion was supported by screening data from relatively small numbers of kinases, and relied on comparisons between different chemical series. This made it difficult to distinguish between differences caused by the binding mode and those due to the compound classes. Incorporating a DFG-out binding group into a DFG-in template changes its selectivity, as was found by others studying Abl inhibitors with DFG-in and DFG-out binding modes.[19] However, these changes are not always for the better. DFG-out BPAs were less selective than DFG-in compounds when tested against an extensive panel of kinases (Table 6).

In conclusion, potent biphenyl amides have been prepared. Crystallography has been used to determine their binding mode and to rationalise the activity in terms of protein conformational flexibility. Minor changes in substitution determine whether p38α is bound in the DFG-in or DFG-out conformation.

The DFG-out binding modes of BIRB-796 to p38α and Gleevec to Abl provoked much interest in this conformation. Comparisons between these molecules and DFG-in inhibitors from different series have revealed differences in selectivity, kinetic and cellular properties. Whilst these may arise from their binding modes, they could simply be properties of the different compound classes. A cleaner comparison can be made between DFG-in and DFG-out compounds from within one series, such as the BPAs presented here. Both DFG-in and DFG-out biphenyl amides are potent ATP-competitive inhibitors of active p38α that bind with similar affinity to inactive p38α. DFG-out binding-mode BPAs achieve greater potency at the expense of higher molecular weight, lower binding efficiency and lower selectivity. The kinetic behaviour of compounds is more complex than 'DFG-in quick, DFG-out slow', depending greatly on the compounds themselves. BPA compounds inhibit the cellular p38α pathway, activation of p38α itself, and cytokine release to a similar extent regardless of their binding conformation. This suggests that some of the perceived advantages of targeting the DFG-out binding mode based on measurements with isolated p38α are not relevant in cells.

Table 5
DFG-in and DFG-out compounds behaviour in cells

Compound	DFG mode	p38α binding[a]	M_W	Binding BEI[b]	p38α activity[c]	HLF Hsp27[d]	HLF p38α[e]	HLF Hsp27/p38α[f]	PBMC TNF-α[g]	PBMC TNF-α/enzyme[h]
SB-242235	In	36	353	21	N.T.	160	130	1.2	170	5
2	In	12	348	23	9	53	18	2.9	250	21
16	In	15	374	21	6	88	16	5.5	650	43
7	Out	2.5	469	18	9	6	2	3.0	90	36
8	Out	4.4	471	18	6	27	6	4.5	270	61
BIRB-796	Out	6.4	528	16	<46	58	47	1.2	30	>5

(a) K_i (nM) for binding to isolated enzyme.[12] (b) BEI (from K_i, not including preincubation, which may be significant for BIRB-796).[12] (c) K_i (nM) for inhibition of enzyme activity.[15] IC_{50} (nM) for inhibition in IL-1-stimulated HLF cells of phosphorylation of (d) Hsp27 and (e) p38α.[24] (f) The ratio between the two HLF readouts. (g) IC_{50} (nM) for TNF-α inhibition in PBMC cells.[12] (h) The ratio between TNF-α IC_{50} in LPS-stimulated PBMC cells and enzyme binding K_i.

Table 6
Kinases inhibited by DFG-in and DFG-out compounds, and the literature results for BIRB-796[4,26]

Compound	DFG	<1 μM	⩾1 μM	KinomeScan[26]
2	In	p38β	–	Only p38β
7	Out	cRAF, p38β	mLCK, LYN	KIT, LCK, LOK, p38β/γ, PDGFRα/β, RAF1, STK10
BIRB-796	Out	JNK2[4]	cRAF, FYN, LCK[4]	JNKs, p38β/γ, SLK, STK10, TEK, TNIK[26]

Acknowledgements

The authors thank Nicola Aston, John Christopher, Duncan Holmes, Rachel Hosking, Gareth Wayne and all members of the p38 team for their many contributions.

References and notes

1. Angell, R.; Bamborough, P.; Cleasby, A.; Cockerill, S.; Jones, K.; Mooney, C.; Neu, M.; Somers, D.; Walker, A. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 318.
2. Angell, R.; Angell, T.; Bamborough, P.; Brown, D.; Brown, M.; Buckton, J.; Cockerill, S.; Edwards, C.; Jones, K.; Longstaff, T.; Smee, P.; Smith, K.; Somers, D.; Walker, A.; Willson, M. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 324.
3. Angell, R.; Aston, N.; Bamborough, P.; Buckton, J.; Cockerill, S.; deBoeck, S.; Edwards, C.; Holmes, S.; Jones, K.; Laine, D.; Patel, S.; Smee, P.; Somers, D.; Walker, A. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 4428.
4. Pargellis, C.; Tong, L.; Churchill, L.; Cirillo, P.; Gilmore, T.; Graham, A.; Grob, P.; Hickey, E.; Moss, N.; Pav, S.; Regan, J. *Nat. Struct. Biol.* **2002**, *9*, 268.
5. Regan, J.; Breitfelder, S.; Cirillo, P.; Gilmore, T.; Graham, A.; Hickey, E.; Klaus, B.; Madwed, J.; Moriak, M.; Moss, N.; Pargellis, C.; Pav, S.; Proto, A.; Swinamer, A.; Tong, L.; Torcellini, C. *J. Med. Chem.* **2002**, *45*, 2994.
6. Regan, J.; Capolino, A.; Cirillo, P.; Gilmore, T.; Graham, A.; Hickey, E.; Kroe, R.; Madwed, J.; Moriak, M.; Nelson, R.; Pargellis, C.; Swinamer, A.; Torcellini, C.; Tsang, M.; Moss, N. *J. Med. Chem.* **2003**, *46*, 4676.
7. Regan, J.; Pargellis, C.; Cirillo, P.; Gilmore, T.; Hickey, E.; Peet, G.; Proto, A.; Swinamer, A.; Moss, N. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3101.
8. Sullivan, J.; Holdgate, G.; Campbell, D.; Timms, D.; Gerhardt, S.; Breed, J.; Breeze, A.; Bermingham, A.; Pauptit, R.; Norman, R.; Embrey, K.; Read, J.; VanScyoc, W.; Ward, W. *Biochemistry* **2005**, *44*, 16475.
9. Dumas, J.; Smith, R.; Lowinger, T. *Curr. Opin. Drug Disc. Devel.* **2004**, *7*, 600.
10. Gill, A.; Frederickson, M.; Cleasby, A.; Woodhead, S.; Carr, M.; Woodhead, A.; Walker, M.; Congreve, S.; Devine, L.; Tisi, D.; O'Reilly, M.; Seavers, L.; Davis, D.; Curry, J.; Anthony, R.; Padova, A.; Murray, C.; Carr, R.; Jhoti, H. *J. Med. Chem.* **2005**, *48*, 414.
11. Schindler, T.; Bornmann, W.; Pellicena, P.; Miller, W.; Clarkson, B.; Kuriyan, J. *Science* **2000**, *289*, 1938.
12. Enzyme K_i determinations, using displacement of a fluorescent ATP-competitive inhibitor, and IC_{50}s for inhibition of TNFα release from LPS-stimulated peripheral blood mononuclear cells were carried out as described in Ref. 2. BEI (Binding Efficiency Index) = (pK_i/M_W)*1000, as defined by Abad-Zapatero, C.; Metz, J. *Drug Discovery Today* **2005**, *10*, 464.
13. Compounds given in Table 2 are representative of an array prepared from substituted benzoic acids as shown in Scheme 2 of Ref. 3, using several amide coupling conditions. Compounds given in Table 3 were prepared as shown in Scheme 1 of Ref. 3, also using a number of different amide coupling conditions. For **22**–**24**, cyclopropylmethyl-amine in step a was replaced with 3-aminobenzonitrile.
14. An *apo* crystal of unphosphorylated human p38α (expressed, purified and crystallised as previously described) was soaked with 2 mM **8** for 5 days and cryoprotected.[1,3] X-ray diffraction data were collected from the crystal at 100 K (using an Oxford Cryostream) at Daresbury Laboratory SRS (station 9.6) using an ADSC Q4R CCD detector. The data were processed with the HKL package (Otwinowski, Z.; Minor, W. *Methods Enzymol.* **1997**, *276:Macromol. Crystallogr. A*, 307) and CCP4 program suite (Bailey, S. *Acta Crystallogr.*, **1994**, *D50*, 760). The structure was solved using the native p38 coordinates (PDB entry 1WFC) as the initial model in refinement by REFMAC (Murshudov, G.; Vagin, A.; Dodson, E. *Acta Crystallogr.* **1997**, *D53*, 240). The final *R*-factor achieved for the complex was 17.5%. Coordinates have been deposited in the PDB as entry 3D83. Figures were produced using Pymol (DeLano, W. L. DeLano Scientific, Palo Alto, CA, USA. http://www.pymol.org).
15. Inhibition of phosphorylation of ATF-2 by activated p38α was measured as described in Ref. 3.
16. Experiments were performed at 25 °C on a BIAcore S51 instrument. Human unphosphorylated p38α (as used in the crystallography) was immobilised using random amine coupling onto a CM5 chip in the presence of SB-203580 at pH 5.3 in 50 mM NaAc at normally 3-4kRU. Compounds were titrated over p38α in 50 mM Hepes pH 7.4, 150 mM NaCl, 1% DMSO, typically in doubling or tripling dilutions from 3.7 μM. BIAcore software and Grafit were used to extract on- and off-rates to ensure consistency and reproducibility.
17. Kroe, R.; Regan, J.; Proto, A.; Peet, G.; Roy, T.; Landro, L.; Fuschetto, N.; Pargellis, C.; Ingraham, R. *J. Med. Chem.* **2003**, *46*, 4669.
18. Lipinski, C.; Lombardo, F.; Dominy, B.; Feeney, P. *Adv. Drug Deliv. Rev.* **1997**, *23*, 3.
19. Okram, B.; Nagle, A.; Adrian, F.; Lee, C.; Ren, P.; Wang, X.; Sim, T.; Xie, Y.; Wang, X.; Xia, G.; Spraggon, G.; Warmuth, M.; Liu, Y.; Gray, N. *Chem. Biol.* **2006**, *13*, 779.
20. Young, P.; McLaughlin, M.; Kumar, S.; Kassis, S.; Doyle, M.; McNulty, D.; Gallagher, T.; Fisher, S.; McDonnell, P.; Carr, S.; Huddleston, M.; Seibel, G.; Porter, T.; Livi, J.; Adams, J.; Lee, J. *J. Biol. Chem.* **1997**, *272*, 12116.
21. Frantz, B.; Klatt, T.; Pang, M.; Parsons, J.; Rolando, A.; Williams, H.; Tocci, M. J.; O'Keefe, S. J.; O'Neill, E. A. *Biochemistry* **1998**, *37*, 13846.
22. Kuma, Y.; Sabio, G.; Bain, J.; Shpiro, N.; Marquez, R.; Cuenda, A. *J. Biol. Chem.* **2005**, *280*, 19472.
23. Ross, S.; Chen, T.; Yu, V.; Tudor, Y.; Zhang, D.; Liu, L.; Tamayo, N.; Dominguez, C.; Powers, D. *Assay Drug Dev. Technol.* **2006**, *4*, 397.
24. Human lung fibroblast (HLF) cells (Lonza, CC-2512) were plated into 96-well tissue culture plates (15,000 cells/well). After overnight incubation, media were replaced with serum-free media. After overnight serum starvation, compounds were added and incubated for 1 h, followed by addition of IL-1α (R&D Systems, 200-LA-010) to a final concentration of 0.09 ng/ml for 40 min. Liquid was removed from the wells, replaced with 40 μl/well Meso Scale Discovery complete lysis buffer, and 35 μl transferred to immunoassay plates for the detection of phospho-p38α (pT180/pY182) or Hsp27 (pS15). Detection and quantification of the level of phosphorylated analyte was performed according to the supplier protocol (MSD), with raw data analysed and IC_{50} values determined using ActivityBase software (IDBS). In addition to BIRB-796, SB-242235 was used as a standard. SB-203580 gave variable results in the HLF assays, perhaps due to its physicochemical properties. For example, from 40 tests in the phospho-p38 assay, it gave $IC_{50} > 10$ μM 7 times and <250 nM 9 times.
25. Adams, J.; Boehm, J.; Gallagher, T.; Kassis, S.; Webb, E.; Hall, R.; Sorenson, M.; Garigipati, R.; Griswold, D.; Lee, J. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2867.
26. KinomeScan assays were carried out at Ambit Biosciences and interpreted as described in Ref. 3. Data for BIRB-796 are taken from Fabian, M. et al *Nat. Biotechnol.* **2005**, *23*, 329.