Bioorganic & Medicinal Chemistry Letters 20 (2010) 5793–5798

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Switch control pocket inhibitors of p38-MAP kinase. Durable type II inhibitors that do not require binding into the canonical ATP hinge region

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ARTICLE INFO

Article history:
Received 25 June 2010
Revised 28 July 2010
Accepted 30 July 2010
Available online 3 August 2010

Keywords:
p38 kinase inhibitor
Type II kinase inhibitor
Switch contol inhibitor
MAP kinase inhibitor
Arthritis

ABSTRACT

Switch control pocket inhibitors of p38-*alpha* kinase are described. Durable type II inhibitors were designed which bind to arginines (Arg67 or Arg70) that function as key residues for mediating phospho-threonine 180 dependant conformational fluxing of p38-*alpha* from an inactive type II state to an active type I state. Binding to Arg70 in particular led to potent inhibitors, exemplified by DP-802, which also exhibited high kinase selectivity. Binding to Arg70 obviated the requirement for binding into the ATP Hinge region. X-ray crystallography revealed that DP-802 and analogs induce an enhanced type II conformation upon binding to either the unphosphorylated or the doubly phosphorylated form of p38-*alpha* kinase.

Discovery and therapeutic development of kinase inhibitors continues to be an area of intense research in oncology, immunology, cardiovascular, and metabolic diseases. The classical small molecule approach to kinase inhibition is based on the design of antagonists to ATP by binding into the ATP cofactor pocket. Such inhibitors bind to kinases which are predominantly in the activated, or so called type I, conformation. Recently, allosteric inhibitors have been reported which bind to kinases that are predominantly in inactive, or so called type II, conformations.[1,2] The seminal reports describing the binding mode of imatinib to ABL kinase and the binding mode of BIRB-796 to p38-*alpha* kinase ushered in this newer era of allosteric type II kinase inhibition.[3,4] Since kinases are more disparate in their inactive conformations, it has been proposed that type II inhibitors hold promise for identifying inhibitors with higher degrees of selectivity.[5] Both type I and type II inhibitors of p38-*alpha* kinase have been reviewed.[6] In this report, we wish to communicate our approach to p38 kinase inhibition based on binding into the p38-*alpha* switch control pocket[7], thus providing a durable mode of type II inhibition that does not require binding into the canonical ATP hinge region, a general requirement for other type II inhibitors.

The conformational state of p38 kinase controls its biological activity. The doubly phosphorylated form of p38 *gamma* (phospho-threonine 180/phospho-tyrosine 182:p38-*alpha* numbering) exists in the prototypical type I catalytically active conformation shown in Figure 1A (taken from PDB 1CM8)[8] while the unphosphorylated form of p38-*alpha* exists in the inactive type II conformation shown in Figure 1B. In the active type I conformation (Fig. 1A), the non-hydrolyzable ATP mimic AMP-PNP occupies the ATP cofactor pocket; in the inactive type II conformation the type II inhibitor BIRB-796 binds allosterically to unphosphorylated p38-*alpha* with a morpholino ring extending into the ATP pocket hinge region, where it makes hydrogen bond interactions with the hinge methionone 109 residue.[4] Of interest to us were specific amino acids which play crucial roles in mediating the conformational fluxing of p38-*alpha* from active to inactive forms. In particular, arginines 67 and 70 from the c-helix, along with arginine 149 from the catalytic loop, play key roles in docking with the phosphorylated war head of phospho-threonine 180 to stabilize the active type I conformation; additionally arginines 186 and 189 dock with the phosphorylated war head of phospho-tyrosine 182 to further stabilize the type I conformation and complete the substrate binding pocket (Fig. 1A). We refer to such positively charged arginines and their negatively charged phosphorylated amino acid binding counterparts as switch control amino acids. Such arginine/phosphate war head interactions are common among kinases, likely reflecting the evolutionary conservation of this mechanism for in vivo kinase regulation.

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doi:10.1016/j.bmcl.2010.07.134

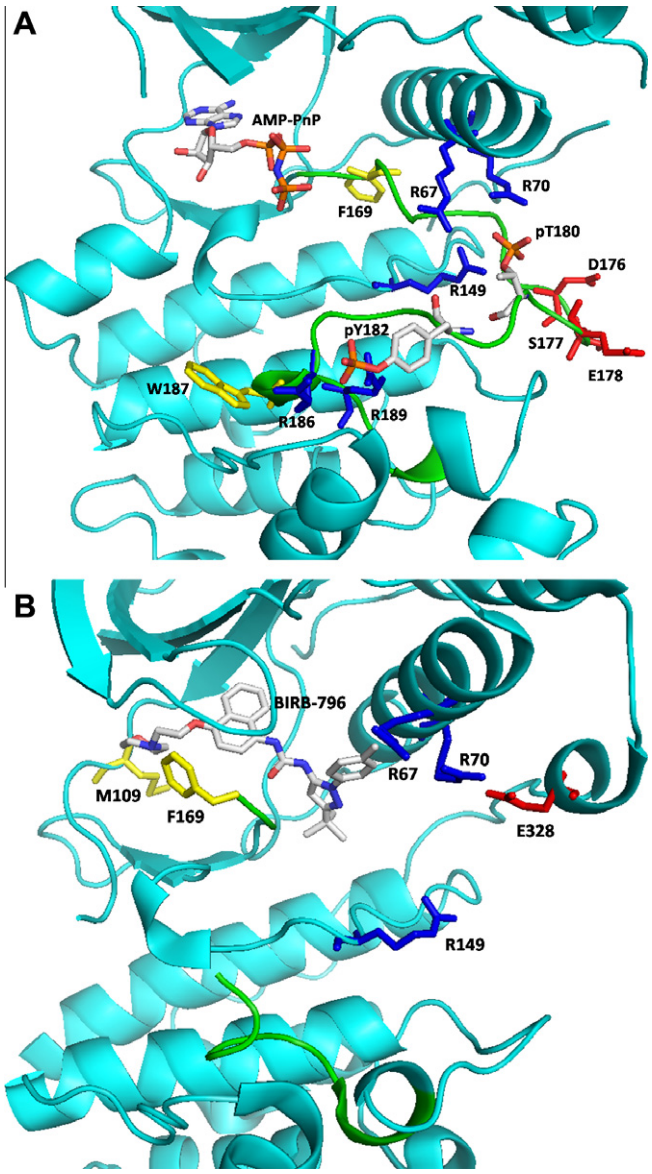


Figure 1. (A) The structure of doubly phosphorylated type I active conformation of p38-*gamma* bound with the ATP mimic AMP-PNP. The activation loop is indicated (green ribbon). Note the electrostatic bonding of phosphorylated Thr180 with the guanidinium warheads of switch control residues Arg67, Arg70, and Arg149; and bonding of phosphorylated tyrosine 182 with the guanidinium warheads of switch control residues Arg186 and Arg189. Taken from PDB 1CM8. P-38-*alpha* amino acid numbering is illustrated. Figure 1(B) The structure of unphosphorylated type II inactive conformation of p38-*alpha* bound with the inhibitor BIRB-796. The activation loop (green ribbon) is only partially represented due to lack of full electron density. Note the alternate bonding of the Arg70 guanidinium warhead with Glu328 from the C-terminal helix. The Arg67 and Arg70 warheads appear available for 'interior-side' bonding by small molecule inhibitors. Phenylalanine (Phe)169 is in the type II inactive state, occluding the ATP pocket.

We were intrigued about the alternative disposition of key arginines 67 and 70 in the inactive type II conformation of BIRB-796 bound to p38-*alpha* kinase (Fig. 1B, taken from PDB 1KV2).[4] In this type II conformation, it is noted that the DFG phenylalanine 169 is in the previously described 'DFG-out' conformation, with the phenylalanine side chain occluding the ATP pocket. Since both threonine 180 and tyrosine 182 are unphosphorylated in this conformation, one or more of the switch control arginines could be available for inhibitor interactions. Indeed in the BIRB-796 type II inactive conformation, the guanidinium warhead of arginine 70 now pairs with glutamate 328 from the C-terminal helix and the warhead of arginine 67 appears to be unpaired with any other amino acid. Both arginine 67 and arginine 70 appear to be available from an interior pocket region continuous with the binding pocket of BIRB-796. We hypothesized that this novel switch-control amino acid binding mode might be useful in inhibitor design, and perhaps reduce or eliminate the requirement for type II inhibitors to anchor in the ATP hinge region for requisite potency. Additionally, since arginine 70 is a fairly unique amino acid in this region of the c-helix, utilization of this residue for inhibitor binding might additionally enhance kinase selectivity.

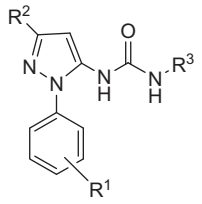
Towards this end, inhibitors were designed and synthesized within the pyrazole urea class which contained extended moieties for binding either arginine 67 or arginine 70. Table 1 illustrates the structures of these initial analogs, along with binding affinities for the unphosphorylated, type II, form of p38-*alpha* kinase. Binding affinities were measured utilizing the fluoroprobe SKF-86002.[4,9] SKF-86002 exhibits a K_d of 180 nM for p38-*alpha* and exhibits fluorescence when bound to p38: emission fluorescence at 420 nm when excited at 340 nm. Direct measurements of binding affinity of inhibitors to unphosphorylated p38-*alpha* can be made by observing a concentration dependent decrease in fluorescence of SKF-86002.

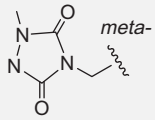
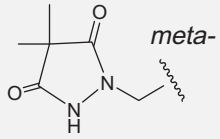
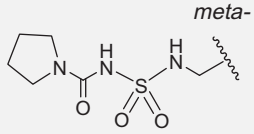
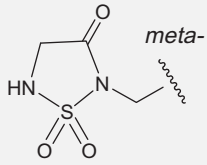
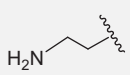
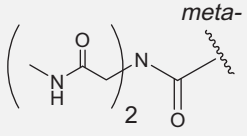
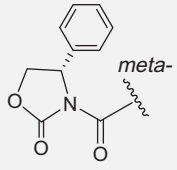
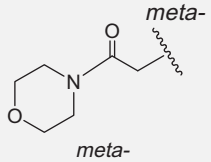
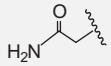
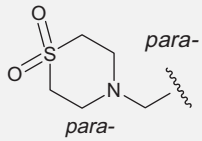
Several different binding moieties were investigated which could potentially engage arginine 70 through hydrogen bond formation. Moieties such as triazolidinedione, pyrazolidinedione, and an acyclic sulfonylurea all proved either weak or modestly potent in this binding assay (entries 1–3). Notably, the *meta*-cyclic sulfonylurea (entry 4) exhibited much increased potency (IC_{50} = 57 nM), indicating a conformational/spatial preference for this moiety over the others. Other *meta*-substituents also afforded modestly potent or inactive analogs, including aminoethyl, disubstituted amide, and acyloxazolidinone (entries 5–7). Of the *meta*-substituents evaluated, acetamides proved to be the most durable and modifiable. The morpholino amide (entry 8) exhibited an IC_{50} of 48 nM, essentially titrating the nominal 80 nM of p38 used in this assay. The simpler unsubstituted acetamide (entry 9) also exhibited an IC_{50} of 68 nM. *Para*-substituted analogs were also evaluated for potential interaction with either arginine 67 or arginine 70. While the aminomethyl analogs were essentially inactive (entries 10 and 11), the hydroxymethyl analog (entry 12) exhibited high potency, IC_{50} = 27 nM. The *para*-propionic acid analog (entry 13) also exhibited high potency, IC_{50} = 50 nM, as did the *para*-thiomorpholino-*S*-dioxide amide (entry 14), IC_{50} = 7 nM. X-ray co-crystal structure determination revealed that an analog of entry 13 bound to p38-*alpha* kinase with the carboxylic acid warhead interacting with arginine 67 and arginine 70 (data not shown). Furthermore, X-ray co-crystal structure determination of entry 12 bound to p38-*alpha* kinase revealed that the hydroxyl moiety forms a hydrogen bond with arginine 70 (Supplementary Fig. S1).

Subsequent structure activity studies further probed the inhibitor series bearing the *meta*-acetamide moiety (Table 2). Further biophysical and biochemical analyses included SKF-86002 fluoroprobe binding studies with both unphosphorylated p38-*alpha* (U-p38) and doubly phosphorylated p38-*alpha* (PP-p38) which is phosphorylated at both threonine 180 and tyrosine 182; thermal melt studies on both the U-p38 and PP-p38 forms, biochemical assessment of potency versus. the PP-p38 form, and off-rate determinations from PP-p38.[10]

The two analogs which contain *t*-butyl at the R^2 position (the moiety for binding into the DFG hydrophobic pocket) afforded potent analogs when evaluated in both biophysical and biochemical assays. In particular entry 16, DP-802, was potent in the SKF-86002 fluoroprobe binding assay, exhibiting IC_{50} values of 9 and 11 nM, respectively, for U-p38 and PP-p38. Thermal melt studies on DP-802 also revealed high stabilization of kinase structure, with ΔT_M values of 12.8 and 9.1 °C for U-p38-*alpha* and PP-p38-*alpha*

Table 1



Entry	R^1arg binding pocket	R^2 DGF binding pocket	R^3 hydrophobic pocket	Fluoroprobe binding assay IC_{50} (nM)
1		Tertiary-butyl	1-Naphthyl	292
2		Tertiary-butyl	1-Naphthyl	384
3		Tertiary-butyl	*p*-Cl-phenyl	1107
4		Tertiary-butyl	*p*-Cl-phenyl	57
5		Tertiary-butyl	*p*-Cl-phenyl	508
6		Tertiary-butyl	1-Naphthyl	949
7		Tertiary-butyl	1-Naphthyl	>10,000
8		Tertiary-butyl	1-Naphthyl	48
9		Tertiary-butyl	*p*-Cl-phenyl	68
10		Tertiary-butyl	1-Naphthyl	2450
11		Tertiary-butyl	*p*-Cl-phenyl	1740
12		Tertiary-butyl	1-Naphthyl	27

(*continued on next page*)

Table 1 (*continued*)

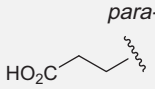
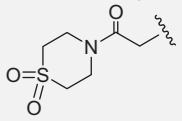
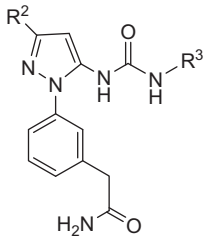
Entry	R^1arg binding pocket	R^2 DGF binding pocket	R^3 hydrophobic pocket	Fluoroprobe binding assay IC_{50} (nM)
13	para- 	Tertiary-butyl	1-Naphthyl	50
14	para- 	Tertiary-butyl	2,3-Cl-phenyl	7

Table 2



Entry	R^2 DFG binding pocket	R^3 hydrophobic pocket	Fluoroprobe binding assay U-p38; PP-p38 IC_{50}, (nM)	ΔT_M[a] U-p38; PP-p38 (°C)	Biochemical IC_{50},nM PP-p38	Koff, min^{-1} and $T_{1/2}$, min PP-p38
15	Tertiary-butyl	1-Naphthyl	16;22	9.1;8.6	7	0.0055 127
16	Tertiary-butyl	2,3-Cl-phenyl	9;11	12.8;9.1	9	0.0024 289
17	Phenyl	2,3-Cl-phenyl	119;79	8.0;5.4	12	0.0164 s42
18	2-F phenyl	2,3-Cl-phenyl	25;40	8.8;7.1	10	>0.01 <5
19	2-Thienyl	2,3-Cl-phenyl	48;66	11.2;8.5	8	0.0117 59
20	3-Thienyl	2,3-Cl-phenyl	15;28	11.6;9.3	6	0.0098 70
21	Cycloptenyl	2,3-Cl-phenyl	5;10	12.2;NT	5	NT

complexes. Entries 15 and 16 also exhibited slow off-rates ($T_{1/2}$ = 127 and 289 min, respectively) when evaluated vs. PP-p38-*alpha*, demonstrating that once bound, these inhibitors likely induce a conformation of PP-p38-*alpha* which requires time dependent conformational fluxing for release of inhibitor. DP-802 (entry 16) was evaluated across a broad range of kinases and was found to inhibit only a narrow range of kinases outside of p38-*alpha* and p38-*beta*, including wildtype BRAF (IC_{50} = 17 nM), CRAF (IC_{50} = 11 nM) and EPHB2 (IC_{50} = 82 nM). Thus arginine 70 offers an inherent selectivity element for binding to the *meta*-acetamide moiety in this inhibitor class.

Further analysis of the distal region of the switch pocket revealed a unique isoleucine 141 at the base of the DFG hydrophobic pocket. This residue is typically a bulkier leucine in other kinases, including BRAF and CRAF kinases. Hence inhibitors with bulkier substituents at the R^2 position (for binding into the deeper isoleucine-containing DFG pocket) were designed to further increase the kinase selectivity of this series (Fig. S2). Phenyl (entry 17), 2-fluorophenyl (entry 18) thienyl (entries 19 and 20), and cyclopentyl (entry 21) moieties were incorporated into inhibitors which resulted in reduced (entry 17) or comparable (entries 18–21) potencies in the p38-*alpha* SKF-86002 fluoroprobe assay. Thienyl analogs (entries 19 and 20) exhibited ΔT_M values for both U-p38 and PP-p38 comparable to DP-802 and also exhibited potent IC_{50} values when evaluated in the biochemical assay (IC_{50} = 8 and 6 nM, respectively). Although the incorporation of 2-fluorophenyl (entry 18) slightly increased the p38 potency versus phenyl (entry 17), the incorporation of 3-fluorophenyl, 3-chlorophenyl, or 4-fluorophenyl at the R^2-position diminished activity in the p38-*alpha* biochemical assay (IC_{50} values of 21, 58, 27 nM, respectively). Similarly, a 3-methyl-2-thienyl analog (IC_{50} = 86 nM) was 10-fold weaker in the p38-*alpha* biochemical assay versus unsubstituted 2-thenyl (entry 19), suggesting that unsubstituted thienyl was the optimal R^2 moiety for binding into the isoleucine 141-lined DFG hydrophobic pocket. Moreover the thienyl analog DP-1376 (entry 19) exhibited an enhanced selectivity profile compared to DP-802. DP-1376 spared BRAF and CRAF (IC_{50} values >5,000 nM) and EPHB2 (IC_{50} >50,000 nM). DP-1376, was evaluated in 151 kinases utilizing the AMBIT KINOMEscan™ kinase profiling platform.[11] Other than its potent inhibition of p38, DP-1376 exhibited moderate affinity (IC_{50} values projected to be >1 μM) for JNK3 and EPHA3 (Fig. S3). The X-ray co-crystal structure of DP-1376 bound to p38-*alpha* kinase is illustrated in Fig. S4. Thus, utilization of p38 kinase switch control pockets provides an approach for potently inhibiting kinase activity and for providing inhibitors with very high selectivity. These attributes are obtained without requirement for inhibitor binding into the classically utilized ATP hinge region.

We solved the X-ray co-crystal structure of DP-802 bound to unphosphorylated p38-*alpha* (U-p38), illustrated in Figure 2A.

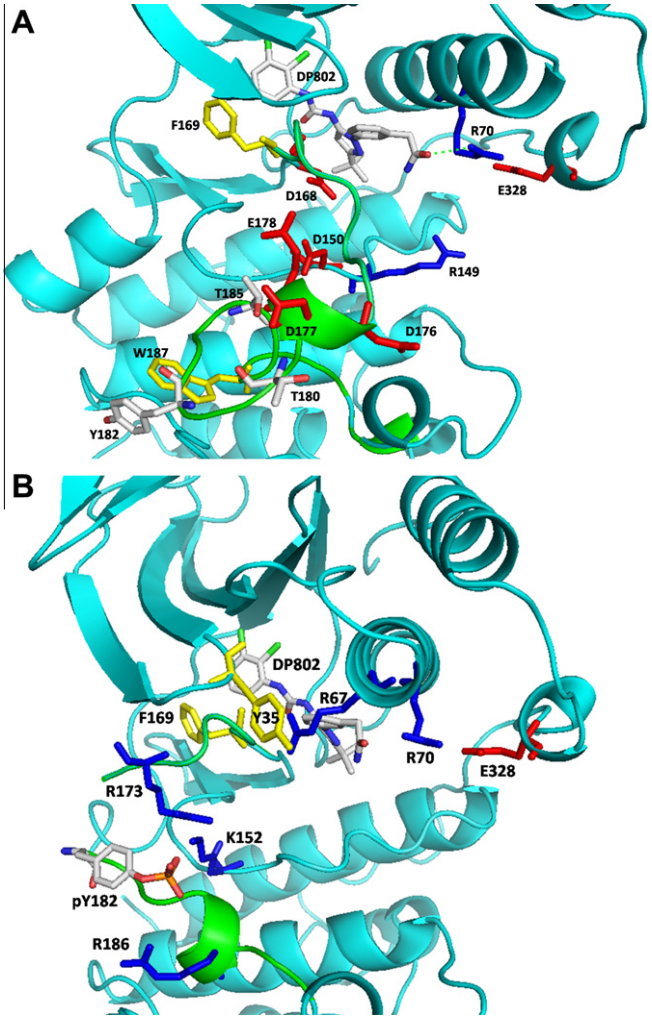


Figure 2. (A) The structure of unphosphorylated type II inactive conformation of p38-*alpha* bound with the switch inhibitor DP-802. The activation loop is indicated (green ribbon). Note the H-bond between the acetamide carbonyl of DP-802 and the 'backside' guanidinium warhead of switch control residue Arg70, while Arg70 also maintains the 'frontside' interaction with Glu328. Tyrosine 35 from the P-loop functions as a switch control residue, stabilizing Arg67 through a face/face π electrostatic interaction. The acidic amino acid cluster of Asp176, Asp177, and Glu178 form a switch pocket for a highly organized H-bond network involving unphosphorylated Thr180, while also positioning of Thr185 for binding as a pseudo-substrate with the catalytic residue Asp150. Trp187 functions as a switch binding site, stabilizing unphosphorylated tyrosine 182 in an edge-face π bonding interaction. (B) The structure of doubly phosphorylated p38-*alpha* bound to DP-802. The activation loop (green ribbon) is only partially represented due to lack of full electron density. Even though this form of p38 is predisposed to a type I active conformation, the inhibitor induces this form to adopt a type II inactive state. The negatively charged Asp176, Asp177, Glu178 likely repel the negatively charged phosphorylated P-Thr180. Hence, it is not possible for this type II state to duplicate that type II state of unphosphorylated p38. There is no electron density identifiable for P-Thr180. P-Tyr182 is stabilized by alternative interactions with Lys152, Arg173, and Arg186.

The *meta*-acetamide carbonyl of DP-802 hydrogen bonds to the back side guanidinium warhead of arginine 70, the urea moiety of DP-802 forms hydrogen bonds with the conserved c-helix glutamate 71/lysine 53 salt bridge, the *t*-butyl moiety occupies the hydrophobic DFG pocket, and the 2,3-di-Cl phenyl ring occupies the type II hydrophobic pocket, stabilizing the DFG phenylalanine 169 side chain in an edge-face π interaction. This binding mode of DP-802 further stabilizes other structural aspects of the type II conformation of p38-*alpha*, which we speculate to be biomimetic of cellular unphosphorylated p38-*alpha*. Indeed, complete electron density is observed through the entire activation loop region allowing detailed analysis of critical elements of the type II off-state of p38-*alpha*. Glutamate 328 stabilizes arginine 70 via a hydrogen bond reinforced ionic interaction; tyrosine 35 from the P-loop extends over to stabilize arginine 67 through an electrostatic guanidinium/aromatic π interaction; aspartate 176, aspartate 177, and glutamate 178 stabilize unphosphorylated threonine 180 through extensive hydrogen bonds and also stabilize threonine 185 for positioning as a pseudo-substrate by forming a hydrogen bond with the catalytic base aspartate 150 from the catalytic loop. Lastly, tryptophan 187 is positioned to intercept and stabilize unphosphorylated tyrosine 182 through an edge-face π interaction. These extensive interactions and conformational restriction of the activation loop likely explain the high ΔT_M value of 13 °C for DP-802 bound to unphosphorylated p38-*alpha*.

We also determined the X-ray co-crystal structure of DP-802 bound to doubly phosphorylated p38-*alpha* (phosphorylated at both threonine 180 and tyrosine 182. Even though doubly phosphorylated p38-*alpha* exhibits a bias for fluxing p38 into its active type I conformation (see Fig. 1A), DP-802 overrides this bias and instead forces phosphorylated p38 into a type II inactive state (Fig. 2B). The structural features for DP-802 binding to phosphorylated p38-*alpha* remain much as for unphosphorylated p38-*alpha* (compare to Fig. 2A). It is evident that significant electron density for regions of the activation loop are now missing. The activation loop cannot resemble that of Figure 1A because the inhibitor preempts the ability of phosphorylated p38 to adopt the type I active conformation. However, this region of the activation loop cannot fully resemble that of the natural type II conformation (see Fig. 2A), because the negatively charged aspartate 176, aspartate 177, and glutamate 178 repel the negatively charged phospho-threonine 180. Indeed there is no electron density evident for phospho-threonine 180, aspartate 176, aspartate 177, or glutamate 178. There is electron density for phospho-tyrosine 182; however, this residue is not paired with arginines 186 and 189 (as shown in Fig. 1A for the type I active state). Rather phospho-tyrosine 182 alternatively pairs with arginines 173 and 186 from the activation loop and lysine 152 from the catalytic loop. Thus, the inhibitor DP-802 induces phospho-tyrosine 182 to identify alternative binding partners consistent with an induced type II inactive conformation.

We hypothesized that such a disordered (phospho-threonine 180) or reordered (phospho-tyrosine 182) orientation of these two switch control amino acids might have an effect on their stability in vivo, and in particular make these two residues more susceptible to in vivo dephosphorylation by cellular phosphatases. Indeed incubation of HELA cells with DP-802 revealed that cellular levels of doubly phosphorylated p38-*alpha* were dose-dependently inhibited, with an IC_{50} value of 19 nM (Fig. 3). Thus, switch control inhibition of p38 kinase may provide therapeutic benefit beyond inhibition

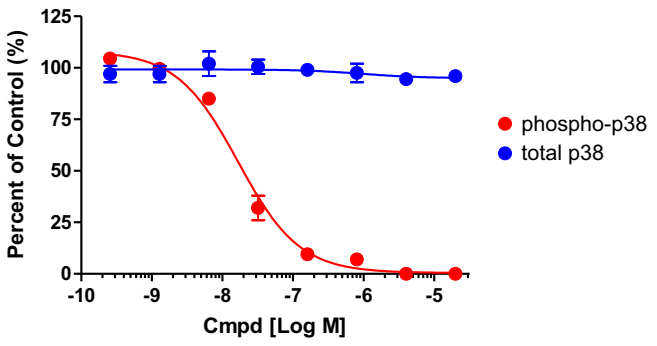


Figure 3. DP-802 induces intracellular de-phosphorylation of p38-*alpha* in HELA cells. The IC_{50} value for DP-802 is 19 nM.

of kinase signaling of p38 per se, and also include contributions such as accelerated dephosphosphorylation by cellular phosphatases, thereby dampening the effects of upstream signaling.

Finally, DP-802 was evaluated in vivo for its ability to inhibit LPS-stimulated TNF-α release in Lewis rats. TNF-α release from circulating peripheral blood mononuclear cells is known to be dependent on p38-*alpha* activity. As illustrated in Figure S5, DP-802 exhibited an ED_{50} of 3.6 mg/kg for inhibiting TNF-α release in Lewis rats at 2 h post dose when given by oral gavage, comparable to an ED_{50} of 4.0 mg/kg exhibited by BIRB-796.

In conclusion, we have described a durable mode of type II kinase inhibition which incorporates inhibitor interaction with key switch control amino acids, that is, arginine 70 in p38-*alpha*. Such a binding mode provides a basis for potent p38-*alpha* biochemical inhibition, slow off-rates for durable in vivo responses, and high kinase selectivity. Furthermore, we have demonstrated that DP-802 induces a type II-like conformation both for unphosphorylated and for doubly phosphorylated/activated p38-*alpha*. The induced type II state for doubly phosphorylated p38-*alpha* demonstrates that a durable type II switch inhibitor is able to control a form of the kinase which otherwise is predisposed to adopt the catalytically active type I state. In HELA cells, it was demonstrated that DP-802 decreased the population of doubly phosphorylated p38-*alpha* in a dose dependent manner, indicating that inhibitor-bound phosphorylated p38-*alpha* is more susceptible to dephosphorylation by cellular phosphatases compared to vehicle control. DP-802 and related analogs exhibit robust in vivo inhibition of p38. Further studies from this effort will be reported elsewhere.

X-ray coordinates have been deposited with the RCSB Protein Data Bank (PDB) for entry 12 in complex with p38-*alpha* kinase (3NNV), entry 16/DP-802 in complex with p38-*alpha* kinase (3NNW), entry 16/DP-802 in complex with doubly phosphorylated p38-*alpha* kinase (3NNX), and entry 19/DP-1376 in complex with p38-*alpha* kinase (3NNU).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.07.134.

References and notes

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