Bioorganic & Medicinal Chemistry Letters 20 (2010) 5787–5792

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Biochemical and biophysical characterization of unique switch pocket inhibitors of p38α

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ARTICLE INFO

Article history:
Received 18 February 2010
Revised 20 April 2010
Accepted 21 April 2010
Available online 28 April 2010

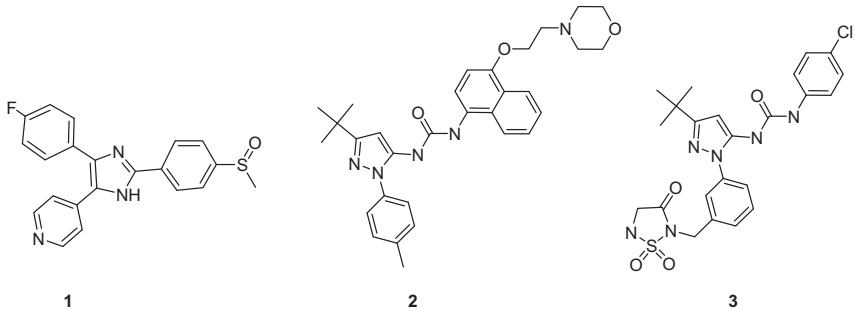
Keywords:
Kinase
Switch pocket
p38
DFG-out

ABSTRACT

Herein we describe the identification and characterization of a class of molecules that are believed to extend into a region of p38 known as the 'switch pocket'. Although these molecules lack a canonical hinge binding motif, they show K_i values as low as 100 nM against p38. We show that molecules that interact with this region of the protein demonstrate different binding kinetics than a canonical ATP mimetic, as well as a wide range of kinome profiles. Thus, the switch pocket presents new opportunities for kinome selectivity which could result in unique biochemical responses and offer new opportunities in the field of kinase drug discovery.

The spatial and temporal control of protein kinases can have significant effects on cell division, growth, development and viability.[1] In terms of drug discovery, kinases offer the potential for modulating a wide range of phenotypic effects in practically every therapeutic area. The past 15 years of kinase drug discovery has clearly shown that the kinase ATP binding pocket is typically a very druggable entity, and direct competition with ATP binding can translate into subnanomolar inhibition of kinase activity. Unfortunately, the homology of the ATP binding site across the kinome makes it very difficult to design selective inhibitors or predict activity across the other members of the kinase family. Also, translating kinase selectivity into a desirable pharmacological and toxicological profile can be very difficult. Thus, although kinases serve as attractive targets for drug discovery, the pleiotropic role of these enzymes continues to translate into significant off-target and toxic effects.

In the past five years, allosteric inhibition of kinases has emerged as an intriguing new approach to drug discovery because targeting domains outside of the ATP binding region, which can have high levels of homology across the kinome, may afford increased selectivity and reduced toxicity. The discovery of molecules that do not bind directly to the canonical ATP site was first



1 2 3

Figure 1. Structures of known p38α inhibitors: (1) SB 203580; (2) Doramapimod (BIRB-796); (3) Deciphera switch pocket inhibitor (Deciphera Pharmaceuticals LLC).

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doi:10.1016/j.bmcl.2010.04.097

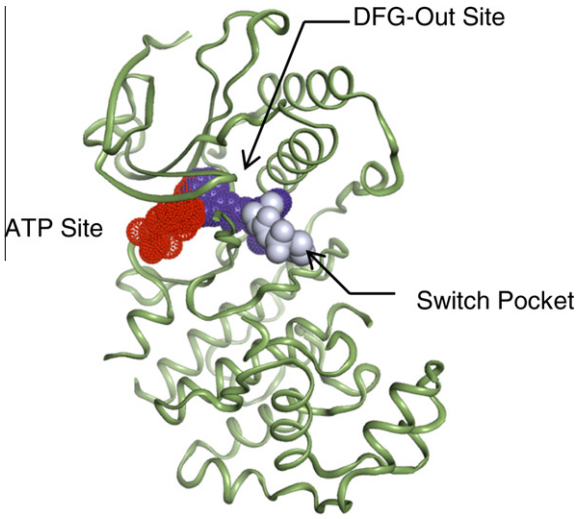


Figure 2. Crystal structure of p38α (1KV2) highlighting the (A) ATP site (red mesh), DFG-out site (dark blue spheres) and switch pocket (light blue spheres).

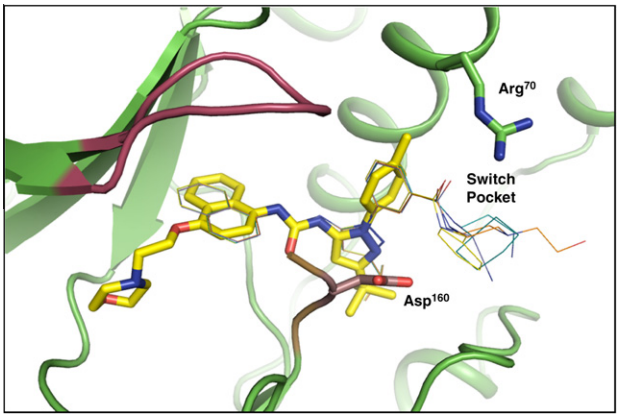


Figure 4. Model of various amide-based switch pocket inhibitors (lines) overlayed with BIRB-796 (yellow sticks) suggesting potential hydrogen bonding interactions with Asp_{160} and Arg_{70} of the switch pocket.

successfully disclosed by Boehringer Ingelheim in 2002 where their Phase IIb/III candidate Doramapimod, a p38α inhibitor, was in development for psoriasis, rheumatoid arthritis and Crohn's disease.[2] This molecule exhibited very good kinome selectivity inhibiting only four other kinases with an IC_{50} less than 1 μM in an in-house kinome panel of 50 kinases. Although no further indication of the progression of this compound has been disclosed, allosteric inhibition of p38 as well as other kinases, was now seen as a viable approach to identifying novel drug candidates. Unfortunately, despite only a few additional reports of non-ATP competitive kinase inhibitors,[3] there is a need for additional biochemical characterization of these types of molecules.

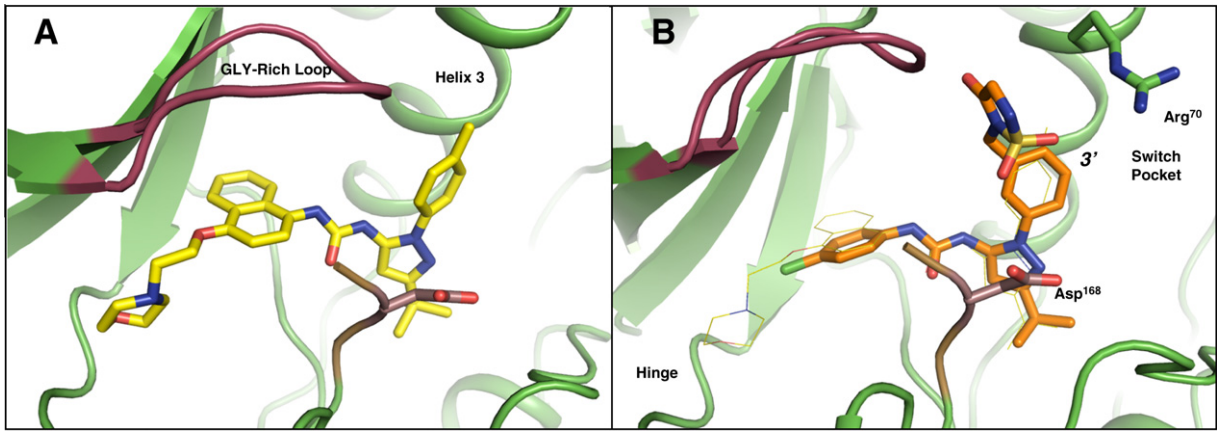
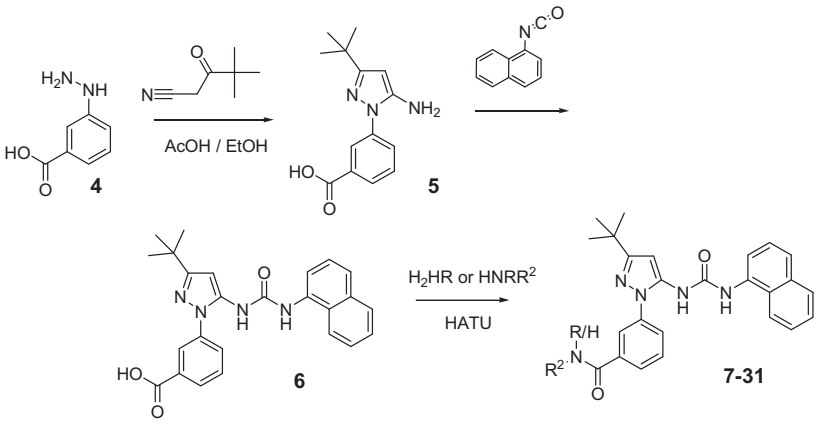


Figure 3. (A) X-ray structure of BIRB-796 in p38α (1KV2); (B) Deciphera switch pocket inhibitor docked in p38α suggesting potential hydrogen bonding interactions with Arg_{70} of the switch pocket.

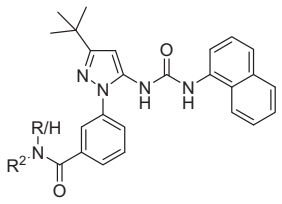


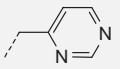
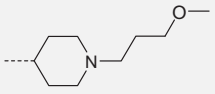
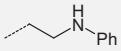
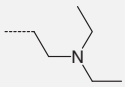
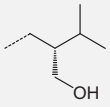
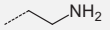
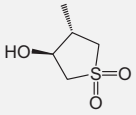
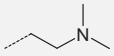
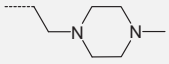
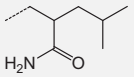
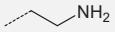
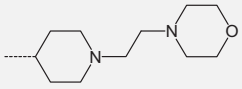
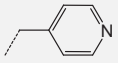
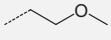
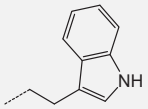
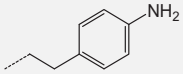
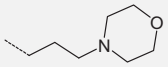
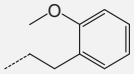
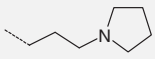
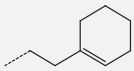
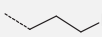
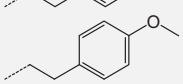
Scheme 1. Synthesis of designed library of p38α switch pocket compounds.[11]

In light of this, there is a clear need to further identify and characterize molecules that bind to distinct allosteric sites outside of the kinase ATP binding site. Here we describe the biophysical and biochemical characterization of a class of small molecules that inhibit p38α by binding to a novel site outside of the canonical ATP binding pocket known as the switch pocket. Although this unique class of compounds lacks a canonical hinge binding motif, some demonstrate low nanomolar inhibition of p38α and exhibit very different binding kinetics, as compared to canonical ATP competitive inhibitors. In addition, interacting with the switch pocket may have unique biochemical consequences as well as present new opportunities for selectivity across the kinome.

P38 kinase, or MAPK14, plays a critical role in the production of proinflammatory cytokines such as TNFα and IL-1β and is activated by a variety of cellular stresses including osmotic shock, lipopolysacchardies (LPS) and growth factors.[1,4] Although there are four highly similar isoforms of p38 (p38α, p38β, p38γ, and p38δ, the alpha isoform is believed to be the key isoform involved in the inflammatory response and thus may offer a novel target for treatment of a variety of diseases including Rheumatoid arthritis and

Table 1
Binding affinities of switch pocket inhibitors of p38α in a TR-FRET assay



Compounds	X	Y	p38α Binding K_i[a] (μM)	Compounds	X	Y	p38α Binding K_i (μM)
SB 203580 (**1**)	—	—	0.057	**18**	H		0.327
BIRB-796 (**2**)	—	—	0.022	**19**	H		0.336
Deciphera (**3**)	—	—	0.099	**20**	H		0.433
7	H		0.086	**21**	H		0.471
8	$-(CH_2)_3NH_2$		0.089	**22**	$-CH_3$		0.715
9	H		0.112	**23**	$-CH_2CH_3$		0.735
10	H		0.140	**24**	H		0.786
11	$-CH_2CH_3$		0.157	**25**	H		0.885
12	H		0.176	**26**	$-CH_2CH_3$		1.39
13	H	$-CH_2CN$	0.252	**27**	H		1.40
14	H	$-(CH_2)_3OH$	0.283	**28**	H		1.45
15	H		0.291	**29**	H		>3.9
16	H		0.301	**30**	H		>3.9
17	$-CH_2(CH_3)_2$		0.312	**31**	H		>3.9

[a] K_i values were calculated using the Cheng–Prusoff equation ($K_i = (IC_{50})/(1 + [probe]/K_d)$).

Crohn's disease. To date, the scientific literature is replete with publications highlighting the discovery and optimization of compounds that inhibit p38α, largely through binding to the canonical ATP site of the kinase. However, this large body of research has yet to yield a successful p38 inhibitor as a marketed drug,[5–7] as a result of either significant toxic side effects or, more recently, a lack of clinical efficacy.

One of the first reported p38 inhibitors was Smith Kline Beecham's SB203580 (Fig. 1-1), whose characterization was first reported in 1993 as a molecule that inhibited the phosphorylation of HSP-27 by a protein kinase, termed at the time, 'reactive kinase (RK)'.[1] In the next 10 years, over 10,000 published articles and patents highlighted the discovery and characterization of other p38 inhibitors that exclusively bind the ATP binding site. In 2002, the report of Boehringer Ingelheim's allosteric inhibitor BIRB-796 (Fig. 1-2) was the first reported kinase inhibitor to extend outside of the ATP binding site, 10 years removed from the characterization of SB 203580 (Fig. 1-1).

Interestingly, two years later, the first in a larger class of patent applications from Deciphera Pharmaceuticals LLC (Lawrence, Kansas)[8] disclosed a novel class of molecules (Example Fig. 1-3) that are believed to extend into a novel allosteric binding site of inactive p38 known as the *switch pocket*. Although these molecules bear a resemblance to the pyrazolyl urea inhibitors originally disclosed by Boehringer Ingelheim, they contain functionality that purportedly extends into a previously unexplored region of the kinase, as well as lack of a canonical hinge binding interaction. The switch pocket is believed to extend into a region which is adjacent to the ATP binding site but partially overlaps with the well known 'BIRB-site' in the DFG-out conformation of the protein (Fig. 2). Interacting with the switch pocket may have unique biochemical consequences as well as present new opportunities for selectivity across the kinome.

Even though public crystal structure coordinates have yet to be made available for inhibitors of this type, the structural similarity of these compounds to the original allosteric BIRB inhibitors indicate a very similar binding mode. In addition, the recent issued patents[8b] around the switch pocket, among other things, indicate that Arginine 70 of helix 3 in the kinase domain plays a critical role in the affinity of these molecules for p38α. This further suggests that these molecules bind to the inactive form of the kinase, and extend into a region similar to BIRB-796. Thus, using the available information in the patent literature,[8] along with the landmark crystal structure of BIRB-796(1KV2), we developed a working model (Fig. 3B) of the switch pocket. This model is depicted in Figure 3B where the pocket is shaped largely by the DFG-loop (tan), the Glycine-Rich- or P- Loop (purple) and helix 3 of the protein. In this particular molecule, the cyclic sulfonylurea is in proximity to ASP_{168} of the DFG-loop, as well as a proximal ARG_{70} on helix 3, both of which may serve as hydrogen bonding partners for the sulfonylurea moiety.

From this, we designed a small library of compounds that would further probe the switch pocket region of p38 (Fig. 4). Our analogs were designed with an amide linker at the 3′ position of the phenyl pyrazole which could potentially maintain H-bonds to the Arg_{70} as well as Asp_{168} of the DFG-loop, and would allow for rapid analoging to explore SAR in this region of the active site. The general synthesis of the proposed analogs is depicted in Scheme 1. Commercially available 3-hydrazinylbenzoic acid (**4**) was treated with pivaloyl cyanide in the presence of acetic acid in ethanol yielding **5** in good yields. The amino pyrazole **5** was then converted to the urea using 1-napthylisocyanate preserving the acid as a handle for further elaboration to a small library of substituted carboxamides.

The focused library of compounds was assayed for their ability bind p38α in a TR-FRET assay[9] along with SB203580(**1**), a competitive ATP site binder, BIRB-796(**2**), and the Deciphera compound(**3**) as control compounds (Table 1). Both SB 203580 and BIRB-796 showed inhibitory activity that reflected their reported literature values of 57 nM and 22 nM, respectively, whereas the representative sulfonylurea analog from Deciphera exhibited a K_i value of 99 nM in our assay. By comparison, the amide based inhibitors exhibited K_i values ranging from 86 nM to greater than 3 μM, depending on the amide substituent. Interestingly, according to this data, the switch pocket seems to be relatively tolerant of various alkyl or aryl substituted amides. However, there is a clear preference for more polar side chains which likely extend into a solvent exposed region of the active site and even potentially form specific interactions with the more hydrophilic portion of the protein. In contrast, larger and more extended hydrophobic compounds tend bind much more weakly to p38, typically showing K_i values above 1 μM.

In light of the fact that molecules that bind the DFG-in and DFG-out conformations of p38α quite often exhibit very different kinetics of binding, we wanted to further evaluate a subset of our compounds using surface plasmon resonance.[10] To this end, the binding kinetics of three compounds with relatively diverse side chains, were evaluated using SPR, along with SB 203580(**1**), a competitive ATP site binder, and the Deciphera compound(**3**). This data is summarized in Table 2.

SB203580, which binds to the canonical ATP binding site demonstrates a 50–200-fold faster on-rate as compared to all of the other compounds in this study. The observed slow on-rate for BIRB-796 is a consequence of the local protein rearrangement

Table 2
Kinetic binding data generated using surface plasmon resonance for two previously reported p38α inhibitors, SB203580(**1**) and BIRB-796(**2**), as well as a representative Deciphera Switch pocket inhibitor(**3**) and 3 amide-based switch pocket inhibitors (**7**, **12**, **13**), against immobilized p38α

	Surface plasmon resonance			TR-FRET
Compounds	K_{on} (1/ms)	K_{off} (1/s)	KD (nM)	p38α Binding K_i (nM)
SB 203580 (**1**)	2138×10^3	.0088	4.1	57
BIRB-796 (**2**)[7]	85×10^3	$.0083 \times 10^{-3}$	.098	9
Deciphera (**3**)	3.7×10^3	.002	564	1360
Compound **7**	56.0×10^3	.0007	14	86
Compound **12**	53.9×10^3	.0005	10	176
Compound **13**	34.4×10^3	.0019	55	250

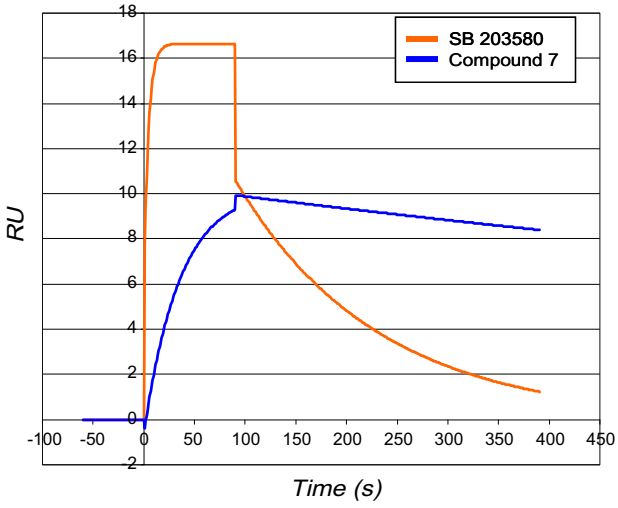
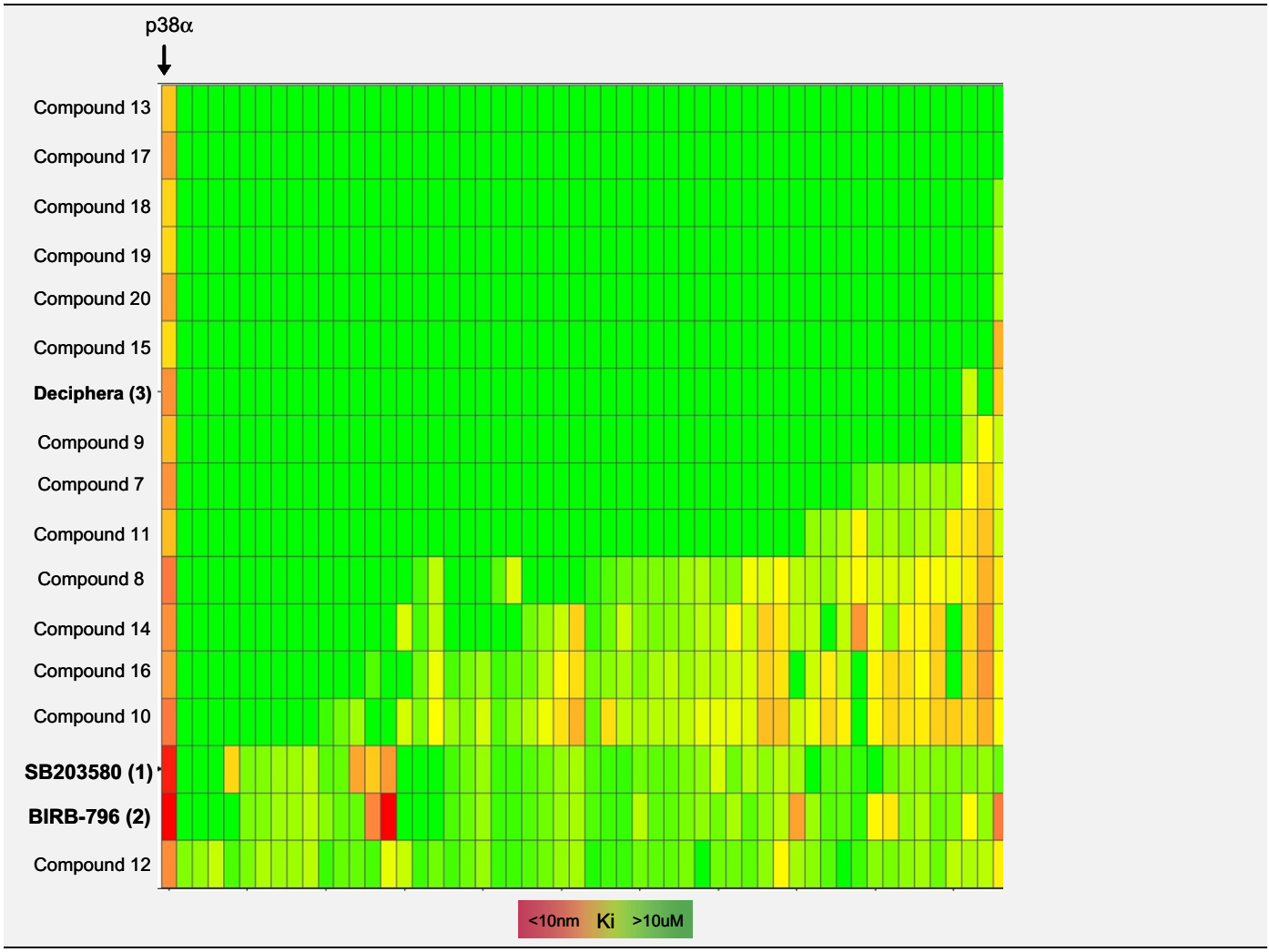


Figure 5. SPR traces of SB203580 (red) and compound **7** (blue) binding to immobilized p38α illustrating different binding kinetics for canonical hinge binders as compared to a representative switch pocket inhibitor.

Table 3
Profile of a subset of 17 switch pocket compounds against a panel of 50 kinases in a TR-FRET assay format



Heat map colors are on a logarithmic scale ranging from red ($K_i \leqslant 10$ nM) to green (K_i >10 μM).

required to adopt the DFG-out form of the kinase. Thus, the fact that the Deciphera inhibitors (**3**) as well as compounds **7**, 12 and **13** demonstrate slower on-rates is additional evidence that these compounds also bind the DFG-out form of p38α.

The SPR traces of SB203580 (red) and compound **7** (blue) are shown in Figure 5 to illustrate how compounds with comparable binding affinities, can have very different binding kinetics. One compelling aspect of this data is the 40× faster on-rate of SB203580 for the kinase, where the protein is completely saturated within 50 s of compound injection. In contrast compound 7 takes twice as long to completely saturate the protein, however, the >10× slower off-rate is quite evident such that the inhibitor is never completely displaced from the protein surface, even after 4 min.

Finally, a subset of our switch pocket inhibitors were then further evaluated against a panel of 50 kinases, along with SB203580, the Deciphera inhibitor (3) and BIRB-796, to further assess the implications of binding to the switch pocket across a representative subset of the kinome. This data is shown in Table 3.

As expected, the kinome profile of SB203580 and BIRB-796 are different, a likely result of the preferential difference in protein conformation. The Deciphera inhibitor seems to be a generally weaker inhibitor, showing only modest inhibitory activity against a few kinases in our assay. By contrast, the kinome profile of the amide-based switch pocket inhibitors can vary dramatically based on the amide side chain. For example, the most non-selective inhibitors, namely compounds **8**, **10**, 14 and **16**, each of which inhibit at least 10 kinases with a K_i value less than 1 μM, seem to exclusively contain amides with basic amines. However, other compounds that are much more selective for p38α (compounds **7**, **9**, **12**, **15**, **17**, **18**, **19**) contain a wide variety of side chains, some of which also include basic amines. Although there seems to be no clear structure–activity relationship to explain the variation in kinome profiles of these compounds, undoubtedly there are opportunities to achieve different selectivity profiles by exploiting this portion of the kinase active site. This is an area of kinase research yet to be explored in any depth.

In summary, we have identified and characterized novel molecules that are believed to extend into a region of p38 known as the switch pocket. Although these molecules lack a canonical hinge binding motif, they still show K_i values as low as from 100 nM. It is clear that molecules that interact with this region of the protein demonstrate different binding kinetics than a canonical ATP mimetic, as well as a wide range of kinome profiles. Thus the switch pocket presents new opportunities for kinome selectivity

which could result in unique biochemical responses and offer new opportunities as in the field of kinase drug discovery.

Acknowledgement

The authors would like to thank Dr. Stevan Djuric for helpful insights and discussions during the course of this work.

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9. TR-FRET competition binding was performed using 2.5 nM p38 Alpha kinase (full-length, GST-tagged protein; Invitrogen cat. no. PV3304), 100 nM tracer 199 (Alexa-647-labeled probe molecule that binds the ATP binding site at a K_d of approximately 64 nM; Invitrogen cat. no. PV5830), 2 nM Europium-labeled-anti-GST antibody (Invitrogen cat. no. PV5594), in a reaction buffer consisting of 50 mM HEPES pH 7.5/10 mM MgCl 2/1 mM EGTA/0.01% Brij-35/0.01% BSA/1 mM dTT. Compounds are tested at different ligand concentrations ranging from 0.0001 to 10 μM in 10× dilutions. Reaction mixtures are incubated for 2.5 h and fluorescence is then measured on a PerkinElmer Envision plate reader. All calculations are performed on the ratio of the two emission values (665 nm from the bound tracer 199', 616 nm from the Eu-anti-GST antibody). Successful competition by the test compound versus the fluorescent probe for the ATP binding site of the kinase is quantified by calculating the EC_{50} value based on a 4 parameter logistic curve fit. A K_i value is then calculated using the Cheng–Prusoff equation ($K_i = (IC_{50})/(1 + [\text{tracer } 199]/K_d)$). Finally', results are visualized in a heatmap which is generated the pK_i values ($pK_i = 6 - \log [K_i]$).
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11. Reagents and conditions: (a) pivaloyl cyanide, A., EtOH, 81%; (b) 1-isocyanatonaphthalene, pyridine, THF, 82%; (c) HATU, DIEA, DMA, amine.