The Discovery of Novel Protein Kinase Inhibitors by Using Fragment-Based High-Throughput X-ray Crystallography

Adrian Gill,* Anne Cleasby, and Harren Jhoti[a]

This article describes the application of a high-throughput X-ray crystallographic fragment-based screening methodology to identify low-molecular-weight leads for structure-based optimisation into protein kinase inhibitors. The identification of two novel p38α MAP kinase inhibitors (with IC_{50} = 65 and 150 nM) starting from low-molecular-weight fragments is described.

Introduction

The phosphorylation of proteins, triggered in response to extracellular signals, represents a fundamental mechanism for the cellular control of many different functions, including gene expression, metabolic pathways, cell growth and differentiation, membrane transport and apoptosis.[1,2] Protein kinases act in concert with cytokines, cell-cycle regulatory molecules, proteins of the apoptotic machinery and transcription factors by pathways that regulate cell metabolism, differentiation, proliferation and death. Many therapeutic strategies are aimed at critical components in signal-transduction pathways, thus, the development of selective protein kinase inhibitors is generating considerable interest in the drug-discovery community.[3–7] To date, over 500 kinase-related sequences have been identified in the human genome, representing approximately 1.7% of our genome.[8]

Protein kinases contain a structurally conserved catalytic domain, first elucidated for PKA, and there are currently over 160 crystal structures of 40 unique protein kinase catalytic domains deposited in the Protein Data Bank (PDB).[9] The vast majority of protein kinase inhibitors under development today are ATP-site-directed inhibitors.[10–13] The high degree of structural conservation in the ATP-binding cleft together with the high intracellular concentrations of ATP with which an inhibitor must compete to generate sufficient cell activity, have historically made the ATP-binding site unattractive as a drug target.

Recently, however, a large number of low-molecular-weight, potent ATP-competitive inhibitors have been identified, many of which show a high degree of selectivity against small panels of kinases (20–30 different kinases).[14–21] In several cases, structural studies clearly show that compounds already known to be selective for a specific kinase or kinase class, target the poorly conserved regions of the ATP-binding site, thus providing a structural basis for the observed selectivity.[22–25] Together, these results have increased confidence that developing inhibitors directed at the ATP-binding site, is a viable approach to selectively inhibit protein kinases.[26]

High-Throughput X-Ray Crystallography

The use of structural information obtained by X-ray crystallography has been a key factor in the design of selective protein kinase inhibitors, and as more structures are solved, the accuracy of the modelling for inhibitor design will ultimately improve.[26,27] Indeed, pharmacophore models for ATP-site-directed competitive inhibitors have been obtained by combining three-dimensional structural information and structure–activity relationship (SAR) data to provide multiple directions for structure-based drug-design approaches.[28–32]

Although the structure of the native target protein can help significantly to guide a lead-discovery program, the maximum value is derived only from structures of the protein in complex with small-molecule inhibitors. This is due to the fact that proteins are conformationally flexible, particularly upon ligand binding, which has proved very difficult to predict from the native structure alone.[33] In addition, water molecules often play a key role in the interactions between small molecules and proteins, and their positions need to be established experimentally. Rapid determination of crystal structures of protein–ligand complexes can effectively guide lead-optimisation programs. Furthermore, high-throughput X-ray crystallography can also be used for fragment-based screening.[34,35]

Some of the first experiments in which X-ray crystallography was used as a screening tool were reported by Verlinde and Nienaber.[36,37] Nienaber et al. described a "CrystalLEAD" screening methodology that focuses on soaking the target crystals with mixtures of fragments with differing shapes, so that they can be easily distinguished by visual inspection of electron density.[38] More recently, a number of groups have described fragment-based X-ray screening approaches towards the identification of Src SH2-binding-domain inhibitors.[39,40] These new fragment-based screening methodologies have been extensively reviewed in a number of recent articles.[27,34,41,42]

[a] *Dr. A. Gill, Dr. A. Cleasby, Dr. H. Jhoti*
Astex Technology
436 Cambridge Science Park, Milton Road, Cambridge, CB4 0QA (UK)
Fax: (+44) 1223-226201
E-mail: a.gill@astex-technology.com

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To transform X-ray crystallography into a screening tool, several technological advances have been necessary.[27,34,41] For example, to facilitate rapid data collection, automatic sample changers, such as the ACTOR system, have been developed.[43] Collection of the X-ray data from a protein crystal exposed to a ligand then needs appropriate analysis and interpretation of the resulting electron density; this requires an objective and automated analysis software. New developments in software, such as AutoSolve® from Astex (Cambridge, UK) and Quanta® from Accelerys (San Diego, USA), can assist in reducing the time taken for data analysis and interpretation. In particular, AutoSolve enables rapid and automated analysis of difference electron-density maps from ligand-soaking experiments by using singlets and cocktails of molecular fragments.[41,44]

Fragment-Based Lead Discovery

Fragment-based lead-discovery approaches aimed at identifying new chemical leads have become increasingly popular over the last five years.[42] The key attractions of fragment-based techniques lie in the synthesis and screening of significantly fewer compounds compared to high-throughput-screening methods, structurally characterised binding modes and the potential high success rate of generating new chemical series with attractive lead-like properties.

Molecular fragments are defined as being compounds in the molecular-weight range of 120–250 Da, that display limited functionality and are therefore expected to exhibit lower affinity in biological assays (30 μM–1 mM range). However, X-ray crystallography can detect low-affinity binding, and the orientations of the molecular fragments observed in resultant crystal structures can guide efficient medicinal chemistry programs to increase potency against a protein target. Fragments can be efficiently linked or grown to generate molecules with increased affinity for the target protein with appropriate lead-like properties (Figure 1).[45]

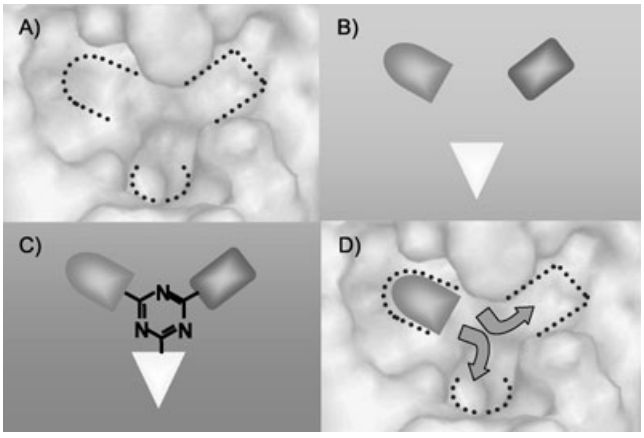


Figure 1. *Structure-based fragment screening. A) A protein with three different binding pockets. B) Structure-based screening can identify molecular fragments that bind into one, two (shown) or all three pockets. C) A lead compound can then be designed by arranging the fragments around a core template, or D) growing out by using iterative structure-based design from a single fragment.*

Another key advantage of using molecular fragments for lead discovery is the significant amount of chemical space that is sampled by using a relatively small library of compounds. For example, if the binding of several heterocycles is probed against specific binding pockets in a protein, the discrimination between a binding and nonbinding event depends solely on the molecular complementarity and is not constrained or modulated by the heterocycle's being part of a larger molecule. This becomes a far more comprehensive and elegant way to probe for new interactions than having the fragments attached to a rigid template, as might derive from a conventional combinatorial chemistry approach.[41] When all the above processes are coordinated they form a rational and powerful approach to lead discovery.

Fragment Libraries

There has been significant literature analysing the relevant properties of small molecules that are required to make good lead compounds.[46,47] Lipinski's "Rule of 5" provides a useful framework for developing orally bioavailable molecules, and these rules have been further developed by Veber, demonstrating that the number of rotatable bonds (NROT) is also an important parameter, with a maximum of 7 seeming optimal for oral bioavailability.[48,49] In addition, there is literature indicating polar surface area (PSA) as another key property. Passively absorbed molecules with PSA $>$ 110–140 Å are reported as likely to have low oral bioavailabilities.[50] More recently, the term "leadlike" was introduced for molecules identified from high-throughput-screening campaigns that are suitable for further optimisation and display properties somewhat "scaled down" from Lipinski values.[47,51] All of these studies address the issues facing compounds discovered by using conventional bioassay-based screening of drug-size compound libraries. Experience of screening low-molecular-weight compounds in our laboratory suggests that a different set of rules applies to fragments.

Table 1 shows the average calculated physico-chemical properties of a set of 40 fragment hits identified against three different targets, one being a protein kinase. Only diverse hits were included in the analysis (in this context, diverse means the hits represent distinctly different opportunities for optimisation). This limited study indicated that the fragments that were hits in the screening process against these targets on average obey a "Rule of 3", in which $M_W < 300$, HBD ≤ 3, HBA $\leq$

Table 1. *Fragment screening hits—average calculated properties.*

Target	No. of Hits	"Rule of 3" properties				Other properties	
		M_W	HBA[a]	HBD[a]	$c\log P$	NROT	PSA
Aspartyl protease	13	228	1.1	2.9	2.7	3.5	44
Serine protease	13	202	1.7	3.1	1.8	2.9	56
Kinase	14	204	2.5	2	1.6	1.7	61
"Rule of 3" Guidelines		<300	≤ 3	≤ 3	≤ 3		

[a] HBA = hydrogen-bond acceptor, HBD = hydrogen-bond donor.

3 and *c*log *P* ≤ 3 (*c*log *P* = calculated lipophilicity).[52] In addition, NROT ≤ 3 and PSA = 60 might also be useful limits for fragment selection. These data imply that a "Rule of 3" maybe a useful tool for constructing fragment libraries for efficient lead discovery.

Different sets of molecular fragments can be used to target a particular protein, based upon diversity or focussed pharmacophore models. For example, family-specific fragment libraries can be assembled, such as a focussed kinase-fragment library. This diverse collection of synthetically tractable chemotypes was based on known ATP-site binders with acceptable leadlike properties (Figure 2). A range of ATP-utilising enzymes (PDE's, ATPases, gyrases, etc), not just protein kinases were analysed to identify a range of literature active-site binders that were fragmented to their core binding motifs. Novel scaffold changes of these literature templates were also incorporated into the set to allow variation of their donor–acceptor–donor motifs, which are well-precedented elements for ATP-site binding.[26,27]

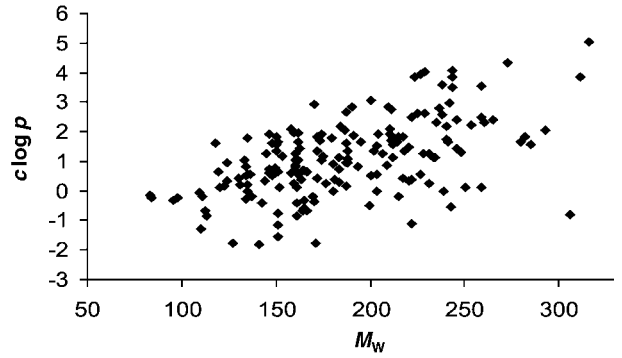


Figure 2. *A scatter graph plotting* c log P *vs. molecular weight for the kinase-targeted library demonstrates that the majority of the library are small, polar molecules that represent ideal starting points as early hits for any kinase-inhibition program.*

Fragment-Based Lead Discovery for p38α MAP Kinase

p38α mitogen-activated protein kinase (MAP kinase) is an intracellular serine/threonine (Ser/Thr) kinase that is activated by a range of environmental stimuli such as TNF-α, IL-1β and stress.[53,54] In its activated state, p38α phosphorylates a range of intracellular protein substrates that regulate the biosynthesis of TNF-α and IL-1β. Excessive production of these cytokines is understood to be significant in mediating the progression of many inflammatory diseases, such as rheumatoid arthritis, Crohn's disease and inflammatory bowel disease.[55–57]

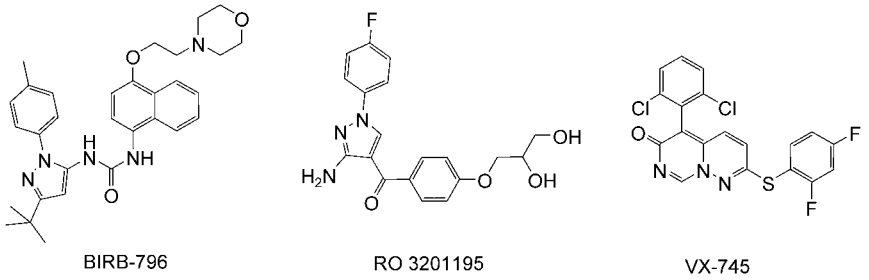
p38α MAP kinase was discovered in the early 1990s, and since then there has been an intense period of drug-discovery activity to identify compounds targeted against it for the treatment of a variety of inflammatory diseases. Currently there are two commercially available anti-inflammatory agents that specifically inhibit TNF-α production. These are Enbrel™ (Etanercept), a soluble TNF-α receptor from Immunex;[58,59] and Remicade™ (Infliximab), a human TNF monoclonal antibody from Johnson & Johnson/Centocor.[60,61] As both of these drugs are administered parenterally, the development of orally bioavailable small-molecule inhibitors of p38α MAP kinase would be greatly preferred in terms of expense, ease of administration and patient compliance.

There has been a plethora of patent and literature publications describing a number of inhibitor templates for p38α MAP kinase, many of which have been expertly summarised in a number of recent reviews.[62–65] Several of the more promising compounds are currently in human clinical trials and have shown good pharmacokinetic and pharmacodynamic properties (Scheme 1). However, there still remains a significant need for orally available small-molecule inhibitors targeting this enzyme.

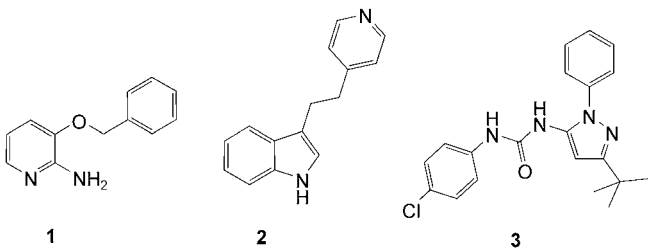
By using high-throughput X-ray crystallographic screening of unphosphorylated p38α with our fragment libraries, the molecular fragments 2-amino-3-benzyloxy pyridine (**1**) and 3-(2-(4-pyridyl)ethyl)indole (**2**) were identified as hits (Scheme 2).[66] These two molecular fragments were then optimised by using structure-guided chemistry approaches to furnish novel, potent and selective p38α kinase inhibitors.[27,66]

Case Study A

Pyridine **1** demonstrated very low affinity for p38α (IC_{50} = 1.3 mM) in an enzyme assay. The crystal structure of p38α complexed with fragment hit **1** was solved to 2.2 Å resolution



Scheme 1. *Structures of p38α MAP kinase inhibitors that have recently advanced into clinical trials.*



Scheme 2. *Structures of Astex fragment screening hits **1** and **2**, alongside competitor diaryl urea **3**.*

(Figure 3) and demonstrates a clearly defined binding mode in which **1** interacts with the hinge region located between the N- and C-terminal lobes of the kinase.[26] The inhibitor makes hydrogen bond interactions through the pyridyl nitrogen to

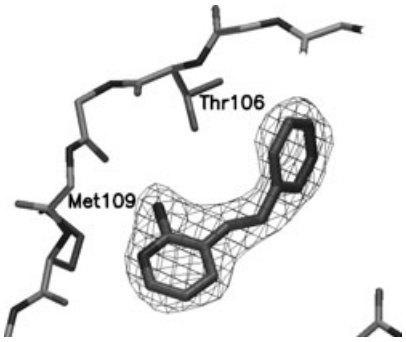
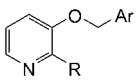


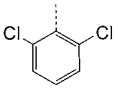
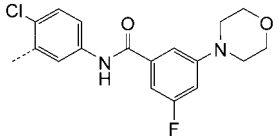
Figure 3. *Omit electron density map for compound **1**. The map is contoured at 3σ. The hinge residue Met109 and the gatekeeper residue Thr106 are labelled.*

the backbone NH of Met109 and the 2-amino moiety to the His107 backbone carbonyl. The benzyloxy group also makes a major interaction by filling the lipophilic specificity pocket (partly created by the gatekeeper residue Thr106) with the side-chain of Lys53 involved in hydrophobic interactions with the phenyl ring.[67]

Synthesis of compound **4** demonstrated that the 2-amino functionality was not essential for activity (Table 2). Analysis of the X-ray structure of **1** suggested that our initial efforts to improve potency of this molecular fragment should focus on improving interactions with the Thr106 lipophilic specificity pocket. Substitution of the phenyl ring of **1** with a 2,6-dichloro substituted aromatic ring (**5**, $IC_{50} = 109$ μM) lead to a tenfold increase in potency over **1**, by virtue of the increased number of hydrophobic contacts in the lipophilic specificity pocket.

Table 2. *Activities of **1** and **4**–**6** against p38α MAP kinase.*



Compound	R	Ar	IC_{50} [μM][a]
SB203580			0.29
3			0.35
1	NH_2		1300
4	H		1030
5	NH_2		109
6	H		0.065

[a] Average of two or more determinations.

The heterocyclic urea work published by Dumas et al. provided some insight into increasing p38α kinase inhibitor activity and selectivity.[68] Indeed, synthesis of urea **3** ($IC_{50} = 196$ nM) and subsequent soaking into p38α crystals revealed a unique binding mode (Figure 4). A conformational rearrangement of

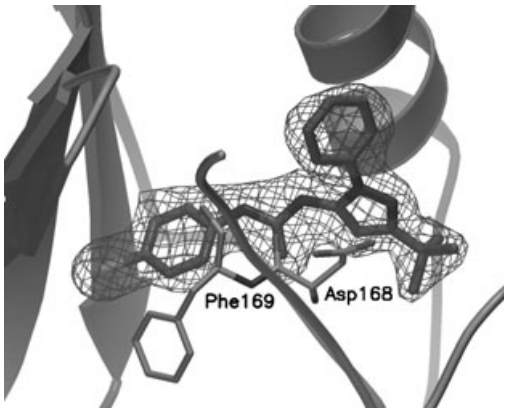


Figure 4. *Omit map for compound **3**, contoured at 3σ, demonstrating that **3** does not interact with the hinge region. The position of the DFG loop is labelled and is in the out conformation.*

the residues Asp168-Phe169-Gly170 (p38α DFG motif) in the conserved activation loop of the kinase was induced and revealed a polar channel formed by Asp168 and Glu71 from the ATP-binding site leading to a lipophilic pocket formed by the approximately 10 Å movement of Phe169.

With this structural insight, a significant potency gain was made by synthesising amide **6** ($IC_{50} = 65$ nM), outlined in Table 2. A large conformational change for the conserved residues of the DFG motif is required for the binding of amide **6** (Figure 5). The Phe169 side chain moves to a new "DFG out" conformation, whereby the Phe169 side chain now covers the

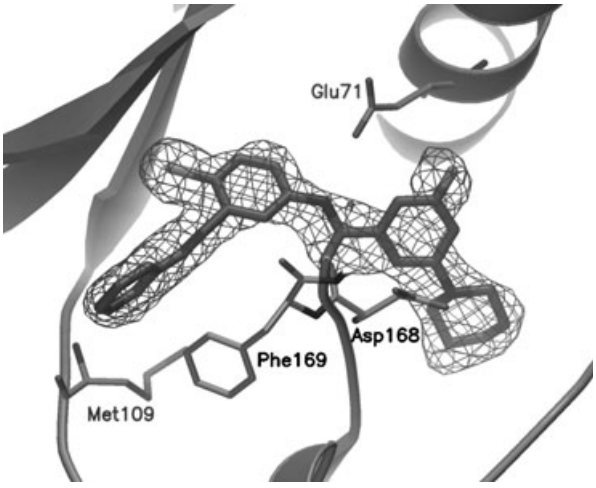


Figure 5. *Omit map for compound **6**, contoured at 3σ. The DFG loop is labelled and is in the out conformation. The hinge residue Met109 is labelled, as is residue Glu71, which forms an interaction with the amide NH of **6**.*

front of the ATP-binding site, effectively locking the kinase in an inactive conformation and rendering it inaccessible to ATP.[69] The polar channel to the allosteric pocket formed by Asp168 and Glu71 is involved in a similar hydrogen-bond network to the amide functionality as observed for urea **3**. This movement of the Phe169 side chain exposes a lipophilic pocket into which the morpholine of amide **6** may then insert. However, most notably, compound **6** still maintains the original anchoring contacts to the hinge and lipophilic specificity pockets.[70]

The selectivity profile of amide **6** against a panel of protein kinases was determined (Table 3). Overall, **6** appeared selective for p38α, especially over the other stress-activated protein kinases JNK-2 and ERK-1. Compound **6** demonstrates over 15 000-fold increase in activity over **1**, achieved by using efficient structure-guided chemistry to make less than 75 analogues in under five months.

Table 3. *Protein kinase selectivity profile of **6**.*

Kinase	Kinase assay IC_{50} [μM][a]
p38α	0.065
JNK-2α2	>10
ERK-1	>10
$P56^{lck}$	>10
CDK-2	>10
MAPKAP-2	>10

[a] Average of two or more determinations.

Case Study B

The molecular fragment **2** demonstrates a second novel and well-defined binding mode to the hinge region of p38α kinase (Figure 6). The inhibitor makes a hydrogen bond through the pyridyl nitrogen to the backbone NH of Met109 and locates the 3-indolyl group in the Thr106 specificity pocket. The indole moiety is buried deep within the Thr106 specificity pocket, with both rings making substantial contacts with a number of neighbouring hydrophobic residues. The indolyl NH acts as a hydrogen-bond donor to the backbone carbonyl of Ala51 (~3.0 Å). Indole **2** is, to the best of our knowledge, the first example of a bicyclic heterocycle-based inhibitor of p38α to bind with the whole of the heterobicyclic ring buried deep within the Thr106 selectivity pocket.

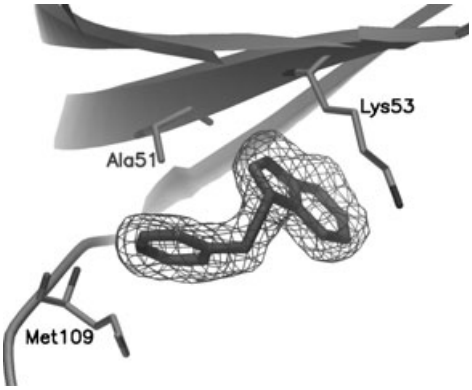
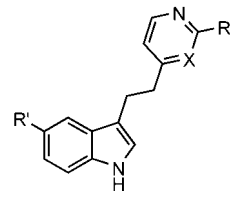
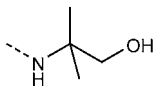
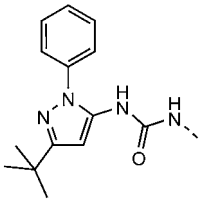


Figure 6. *Omit map for compound **2**. The difference electron density is contoured at 3σ. The hinge residue Met109 and the residues Ala51 and Lys53 are labelled.*

Indole **2** is a competitive inhibitor with respect to ATP and also demonstrated greater affinity for p38α (IC_{50} = 35 μM) than **1** (Table 4). Analysis of the X-ray structure suggested that our initial efforts to improve potency should focus on improving the interactions with the hinge region of the ATP-binding site. The diarylimidazole-based p38 inhibitors have been shown to display improved potency and selectivity by amino substitution at the 2-position of the key Met109-binding heteroaryl residue. From a small range of 2-amino heterocyclic substitutions, the 2-amino-2-methylpropanol derivative **7** was found to be particularly active (Table 4, IC_{50} = 1.5 μM) giving an over 20-fold increase in potency compared to **2** with little increase in molecular weight (310 Da).

Table 4. *Activities of **2** and **7–8** against p38α MAP kinase.*



Compound	R	R′	X	IC_{50} [μM][a]
SB203580				0.29
3				0.35
2	H	H	C	35
7		H	N	1.5
8	H		C	0.15

[a] Average of two or more determinations.

A further increase in p38α activity was gained with the synthesis of compound **8** (Table 4, IC_{50} = 150 nM), targeted to interact with the DFG region, akin to compound **6**. By preparing only 20 compounds using structure-guided chemistry, we successfully generated compounds, such as urea **8**, that displayed over 250-fold improvement in activity over **2**.[70]

Conclusion

The role of protein structure in developing novel protein kinase inhibitors will increase significantly over the coming years as more crystal structures become available. Many recent technological advances in structure determination have al-

lowed X-ray crystallography to be used as a method for ligand screening.[34,41,43] This has significantly increased its use in fragment-based lead discovery in which the initial molecular fragments are likely to have an affinity too weak to enable detection by using traditional enzyme-assay-based methods.

A key strength of fragment screening by high-throughput X-ray crystallography lies in its potential to use less-complex molecular starting points and to identify areas of chemistry space that have not previously been exploited. A strong intellectual property position continues to be a necessity for undertaking an expensive drug-discovery campaign, and this methodology represents a new opportunity to explore chemical structures that are not well represented in corporate collections or suppliers' catalogues. This is particularly important in the protein kinase inhibitor arena, in which currently many inhibitor scaffolds are based around a limited number of well-documented heterocyclic templates.[27]

Using a fragment-based approach, we have identified novel, potent and selective series of p38α kinase inhibitors starting from the low-affinity hits **1** and **2** and using structure-guided chemistry approaches. The synthesis of analogues of **1** and **2** is simple and amenable to large scale, allowing for the rapid production of a wide range of derivatives. Compound **6** demonstrates over 15000-fold increase in affinity over **1** and was identified in under five months and by synthesising fewer than 120 analogues. Compound **6** also displays drug-like physicochemical properties and excellent selectivity over a small panel of kinases. From compound **2**, preparing only 20 compounds by structure-guided chemistry, led to the successful generation of urea **8**, which displayed over 250-fold greater activity. Based on these results, high-throughput fragment-based X-ray crystallographic screening shows great promise as a new approach for the discovery and optimization of novel protein kinase inhibitors.

Experimental Section

p38α kinase enzyme assay: An enzymatic assay was developed in-house: p38α (17 μg, produced in-house) was activated overnight by MKK6 (0.06 μg, Upstate Discovery) in HEPES (25 mM, pH 7.4), β-glycerophosphate (25 mM), EDTA (5 mM), $MgCl_2$ (15 mM), ATP (100 μM), sodium orthovanadate (1 mM) and 1,4-dithiothreitol (DTT; 1 mM). Activated enzyme (diluted to 10 nM) was incubated with myelin basic protein (5 μg) in HEPES (25 μL, 25 mM, pH 7.4), β-glycerophosphate (25 mM), EDTA (5 mM), $MgCl_2$ (15 mM), ATP (70 μM), sodium orthovanadate (1 mM), DTT (1 mM) and $^{33}P\chi$-ATP (0.35 μCi) for 50 min. Compounds and controls were included in a DMSO concentration of 10%. The reaction was stopped by the addition of 2% orthophosphoric acid (30 μL) and transferred to Millipore MAPH filter plates, prewetted with 0.5% orthophosphoric acid (50 μL). The plates were filtered and washed twice with 0.5% orthophosphoric acid (200 μL). Incorporated radioactivity was measured by scintillation counting and IC_{50}s were calculated from replicate curves by using GraphPad Prizm software.

Kinase selectivity data outlined in Table 3 were obtained from Upstate Discovery.

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Keywords: drug design · kinase inhibitors · lead generation · MAP kinase · screening · X-ray crystallography

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