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SAR and inhibitor complex structure determination of a novel class of potent and specific Aurora kinase inhibitors

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Abstract—A novel series of 5-aminopyrimidinyl quinazolines has been developed from anilino-quinazoline **1**, which was identified in a high throughput screen for Aurora A. Introduction of the pyrimidine ring and optimisation of the substituents both on this ring and at the C7 position of the quinazoline led to the discovery of compounds that are highly specific Aurora kinase inhibitors. Co-crystallisation of one of these inhibitors with a fragment of Aurora A shows the importance of the benzamido group in achieving selectivity.

The Aurora family of serine/threonine protein kinases is critical for the proper regulation of mitosis. Mammals express three Aurora kinase paralogues, and at least two Aurora kinases (Aurora A and B) are commonly overexpressed in human tumours.[1] The Aurora A gene is amplified in many tumours, indicating that overexpression of Aurora A may confer a selective advantage for the growth of these tumours.[1] Recent clinical experience and subsequent approvals of small-molecule kinase inhibitors such as Imatinib,[2] Gefitinib[3] and Erlotinib[3] illustrate the tractable nature of this class of enzymes for pharmaceutical intervention, in particular for anti-cancer drug development. Aurora A itself has been identified as a particularly attractive drug target through observations that it can act as an oncogene and transform cells when ectopically expressed.[1] Encouragingly, VX-680, a potent and specific inhibitor of Aurora A and B kinases, has been shown to suppress tumour growth in vivo and this agent has now progressed into clinical trials.[4] These findings underline the desirability of identifying small-molecule Aurora kinase inhibitors and evaluating their potential value for the treatment of human cancers.[5] In this paper, we report the development of a highly potent series of specific Aurora kinase inhibitors and describe the X-ray structure of such an inhibitor bound to a fragment of Aurora A kinase.

In an effort to discover novel Aurora kinase inhibitors, we screened the AstraZeneca compound collection against Aurora A kinase. Anilino-quinazoline **1**[6] was identified as an interesting starting point for further optimisation. This compound showed excellent levels of Aurora A enzyme inhibition[6] and was also effective in an MCF7 cellular anti-proliferative assay[6] (see Table 1).

Clear SAR was evident from the primary screening data and suggested that compounds, which contained a large *para*-substituent on the aniline, showed good specificity towards the Aurora kinases.

Our initial exploration of SAR around the quinazoline moiety resulted in the discovery of compound **2**,[6] where the methoxy group at the C7 position of the quinazoline has been replaced by a 3-(1-morpholino)propoxy side chain. This compound exhibited much improved cellular potency when compared to our initial hit.[7] Evaluation of compound **2** in a panel of in vitro kinase inhibition assays demonstrated that it possessed good activity

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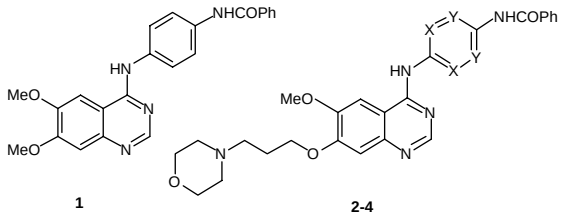
Table 1. In vitro SAR of quinazoline leads[a]



Compound	X	Y	Aur A IC_{50} (nM)	MCF7 cell IC_{50} (nM)	Log D[b]
1			393	1250	3.7
2	C	C	110	198	3.5
3	C	N	3	210	2.7
4	N	C	629	—	

[a] With the exception of compound **4**, all IC_{50} values represent averages of at least two experimental results.
[b] Measured at pH 7.4.

against the Aurora kinases (Aurora A IC_{50} = 0.11 μM, Aurora B IC_{50} = 0.13 μM). In contrast, the compound showed only modest activity against other kinases (MEK1 IC_{50} = 1.79 μM, Src IC_{50} = 1.03 μM, Lck IC_{50} = 0.88 μM) and no activity was seen (>10 μM) against CDK1, CDK2, CDK4, PLK1, CHK1, IKK1, IKK2 and FAK.[7]

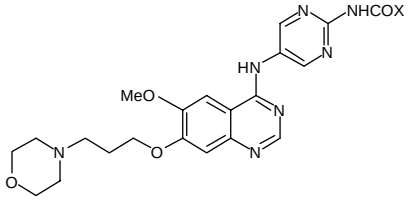
Although both potent and specific, compound **2** suffered from high lipophilicity, resulting in poor physiochemical properties (aqueous solubility: 2.5 μM, rat plasma protein binding: 0.3% free). In an effort to improve these physical properties, we investigated strategies to lower the log D by replacing the central aniline with amino heterocycles, such as pyrimidine. It was observed that the 5-pyrimidyl analogue (compound **3**, Table 1) did indeed have lower lipophilicity (log D = 2.7, compared with compound **2** with log D = 3.5) resulting in much improved levels of free drug (rat plasma protein binding: 4.5% free), although the compound's aqueous solubility remained poor. As well as the beneficial effect on free drug levels, the compound showed an unexpected improvement in enzyme potency that was found to be highly sensitive to which isomer of the pyrimidine was employed. Thus, the 5-pyrimidinyl isomer (compound **3**) is approximately 200 times more potent against Aurora A than the 2-pyrimidyl isomer (compound **4**).[8]

Using the 5-aminopyrimidinyl analogue (compound **3**) as our new lead, we next investigated the effect of substitution on the phenyl ring of the benzamido group. Aurora A enzyme potency data suggested a strong preference for small, lipophilic substituents. Thus, the 3-chloro and the 3-chloro, 4-fluoro analogues (compounds **5** and **6**) gave excellent levels of enzyme potency which also resulted in a significant improvement in cell activity (80 and 20 nM, respectively). The introduction of larger groups (compounds **7** and **8**) had a detrimental effect on potency, whilst introduction of a very large sulfonamido group (compound **9**) resulted in a 1000-fold drop in activity when compared with compound **3**. Replacement of the phenyl ring with either a heterocyclic group (e.g., 4-pyridyl, compound **10**) or an alkyl substituent (e.g., *n*-butyl, compound **11**) gave compounds with much improved aqueous solubility but the drop in potency against both enzyme and cell assays was unacceptable (Table 2).

Although beneficial for potency, the introduction of the halogens on the pendant phenyl ring resulted in increasingly lipophilic molecules displaying disappointing levels of aqueous solubility and plasma protein binding (e.g., compound **6** showed 1.4 μM solubility and only 0.4% free drug in rat plasma). Early modelling studies, later supported by the X-ray structure of the enzyme: inhibitor complex, indicated that the C7 substituent on the quinazoline lay in a channel that placed the morpholine in a solvent-accessible region. Further refinement of the C7 substituent demonstrated that whilst substitution was not tolerated on the methylene unit nearest to the quinazoline, there was considerable scope for introducing solubilising groups at the far end of the chain. Compound **12** (Fig. 1), where the morpholine group had been replaced with the more basic piperidine moiety, has a dramatically improved level of aqueous solubility (3600 μM). Furthermore, it was clear that neither the Aurora A enzyme potency (0.8 nM) nor the activity of these compounds in the MCF7 anti-proliferative assay (24 nM) was affected by these modifications. The introduction of more polar hydroxyl groups also gave very soluble compounds (compound **13**, aqueous solubility: 1500 μM). With highly soluble and specific Aurora kinase inhibitors in hand, we were able to attempt to obtain a crystal structure of such a compound bound to Aurora A.

Different crystal forms (Table 3) were obtained for Aurora A in complex with a non-hydrolysable ATP analogue (ADPNP)[9] and with compound **13**.[10] A T287D mutant intended to mimic the phosphorylated (activated) kinase and to limit heterogeneity in phosphorylation

Table 2. In vitro SAR around benzamide substitution[a]



Compound	X	Aur A IC_{50} (nM)
5	3-Chlorophenyl	<0.1
6	3-Chloro-4-fluorophenyl	0.15
7	3-Bromo-4-methylphenyl	70
8	4-Ethylphenyl	85
9	(4-Dipropylaminosulfonyl)phenyl	3900
10	4-Pyridyl	690
11	*n*-Butyl	17

[a] All IC_{50} values represent averages of at least three experimental results.

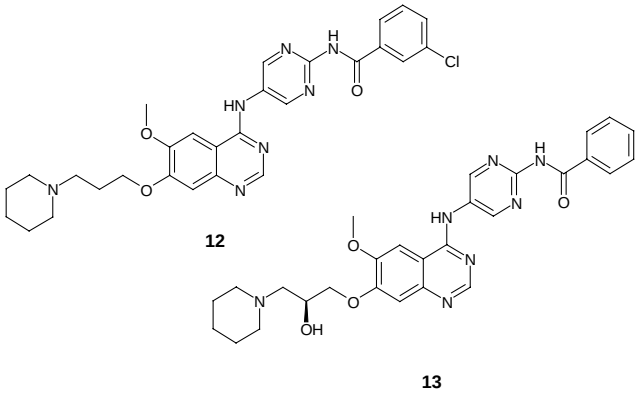


Figure 1. Soluble Aurora inhibitors.

Table 3. Crystallographic data and refinement statistics

	Aurora-ADPNP	Aurora-compd **13**
Space group	$P3_221$	$P2_1$
Unit cell	86.5, 86.5, 78.3 Å	52.6, 88.4, 67.8 Å
Parameters	90, 90, 120°	90, 90.01, 90°
Total reflections	62,278	36,664
Unique reflections	17,003	26,294
R_{sym}[a]	3.6% (32.6%)	6.6% (30.5%)
Resolution	38–2.2 (2.25–2.2) Å	53–2.1 (2.25–2.2) Å
$I/\mathrm{sig}I$	19.3 (3.0)	7 (2.1)
Completeness	97.1% (83.2%)	72.5% (25.5%)
$R_{(free)}$, $R_{(work)}$	23%, 28%	22%, 27%
rmsd bonds	0.006 Å	0.019 Å
rmsd angles	1.2°	1.8°

[a] Numbers in parentheses refer to data in the highest resolution shell.

state was used. Limited proteolysis defined a suitable construct of the kinase domain for crystallisation.[11] Cyclic-AMP kinase (PKA)[12] was used as a trial model to solve the structures by molecular replacement,[13] since currently available Aurora crystal structures[14–17] had not yet been reported. The ADPNP complex and the two unique molecules in the compound **13** complex display many small structural differences, illustrating the inherent flexibility of the kinase. Being able to apply parts of the model from one structure to the other during concurrent rebuilding allowed clarification of regions of electron density that would otherwise have been difficult to interpret, and the ADPNP structure contributed significantly to the solution of the compound **13** complex.

The structures[18] show the bilobal shape characteristic of protein kinases with the ATP- and inhibitor-binding sites situated between the two lobes (Fig. 2a). The activation loop, residues 279–290 containing the T287D mutation, is disordered in both structures suggesting mobility, as is the case in many other kinase structures. The structures adopt a conformation typical of catalytically inactive kinases, despite the introduction of the activating mutation. We considered whether the acidic pH of crystallisation of the ADPNP complex might result in protonation of the introduced aspartic acid such that it no longer mimics the phosphorylated threonine in the wild-type activated protein. Indeed, kinase activity for the mutant falls as the pH is lowered (data not shown). This effect seems unlikely to be the only contribution, since recent reports indicate a binding partner helps achieve fully active kinase for both Aurora A[16] and B.[17] The inactive conformation is clearly capable of binding compound **13** and the ATP analogue, and is useful for structure-based design.

The ADPNP molecule is modelled in two conformations that differ mainly in the phosphate orientations, and the γ-phosphate is disordered. One orientation corresponds to that normally seen; we do not attach any physiological relevance to the other which might simply be a consequence of having no Mg^{2+} in the crystallisation buffer. Compound **13** binds in the ATP-binding site in the cleft between the two domains (Fig. 2a). It adopts an extended conformation, demonstrating the extent of the available binding pocket. The N-terminal lobe helix (residues 174–182) is further away from the ATP-binding pocket compared with the equivalent helix in PKA, extending the relative length of the cleft between the two lobes in Aurora, such that Aurora may be able to accommodate longer inhibitors. This may explain the early SAR observed on our screening output, that a large *para*-substituent on the aniline was required for potency and specificity. A classical kinase (adenine-mimetic)

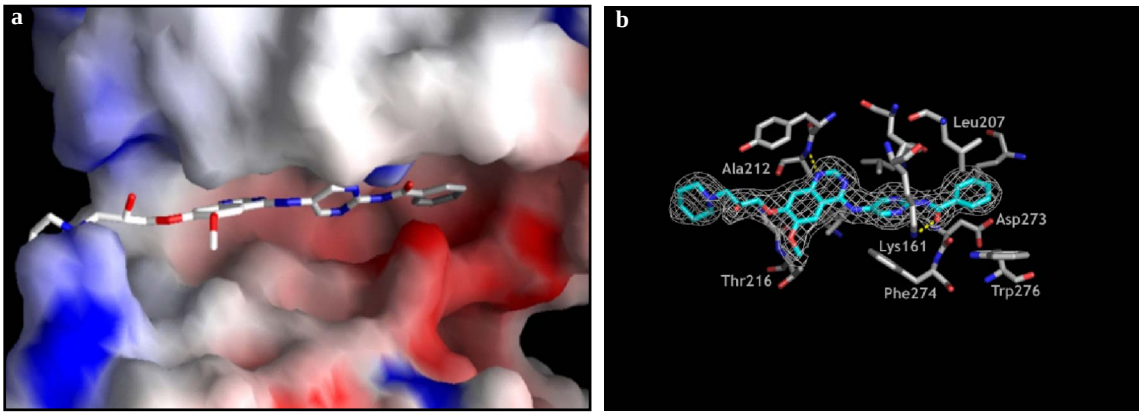


Figure 2. Crystal structure of Aurora A with compound **13**. (a) Surface diagram indicating the extent of the cleft between the N and C-terminal lobes and the fit of the elongated inhibitor molecule; (b) the inhibitor in final $2F_o - F_c$ electron density, indicating hydrogen bonds with Ala212 and Lys161. The conserved DFG motif in the activation loop adopts a 'DFG-out' conformation.

inhibitor hydrogen bond interaction with the main chain peptide is made between N (17) in the inhibitor and the amide of Ala212 (Fig. 2b). The piperidine moiety of the inhibitor extends into solvent. The C4-aminopyrimidine ring is nearly co-planar with the benzoyl group but tilted with respect to the quinazoline. The amide carboxyl between the pyrimidine and benzoyl rings shows a hydrogen bond to the conserved Lys161, while there are water-mediated contacts between the protein and the amide nitrogen. The benzoyl moiety fits into a hydrophobic pocket, which is occupied by Phe274 in the conserved DFG motif in the ADPNP structure. The inhibitor stabilises a 'DFG-out' conformation, which typically prevents the activation loop from adopting a productive conformation for activation by phosphorylation and may contribute to the inhibitory mechanism. Possibly the inhibitor selects the inactive DFG-out conformation also in crystallisation. These structures have aided numerous insights into Aurora inhibitor design.[5]

In summary, we have developed a series of novel and potent kinase inhibitors which show excellent levels of specificity for Aurora kinases. In addition, we have been able to solve the X-ray structure of one of these inhibitors bound to Aurora A, resulting in a clearer understanding of the SAR for this class of inhibitors.

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9. Crystallisation at 15 °C by hanging-drop vapour diffusion used 10 mg/mL protein (in 40 mM HEPES, pH 7.4, 2 mM DTT and 50 mM NaCl), 5 mM ADPNP added, incubated on ice for 30 min, microfuged for 10 min and then mixed in a 1:1 ratio with reservoir buffer (0.2 M K_2HPO_4, 1.6 M NaH_2PO_4 and 0.1 M phosphate/citrate, pH 3.8) to give 4 μL drops. Crystals were transferred for 20 s to 0.2 M K_2HPO_4, 1.6 M NaH_2PO_4, 0.1 M phosphate citrate, pH 3.8 and 30% glycerol before being cooled to 100 K in a nitrogen gas stream.
10. Crystallisation used 10 mg/mL protein, 40 mM HEPES, pH7.5, 50 mM NaCl and 1 mM 2-mercaptoethanol added to 5 mM inhibitor, in a 1:1 ratio with reservoir solution (22% PEG 4000 and 0.2 M ammonium sulfate). Crystals have two molecules per asymmetric unit. Data were collected at room temperature; the crystal could be translated to allow multiple exposures. Data for both structures were collected at beam line PX9.6 at the SRS, Daresbury, on an ADSC Quantum4 CCD detector and were analysed using CCP4 software.[19]
11. The region from amino acid 94 to the stop codon was amplified by PCR to provide MG-6His-GS-[T287D]Aurora A(94-402), but the resultant protein was poorly soluble. Limited proteolysis with endoproteinase GluC from *Streptococcus aureus* V8 (Boeringer Mannheim UK, Lewis, Sussex, UK) produced sufficient quantities of purified product (affinity chromatography followed by cleavage and size exclusion), identified by LC-ESMS and N-terminal sequencing as [T287D]Aurora A(122-396), and yielded crystals of the ADPNP complex. Recombinant protein (GSMH-[T287D]Aurora A(122-400), after thrombin cleavage of a 6His tag) yielded crystals of the inhibitor complex.
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