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The synthesis and SAR of 2-amino-pyrrolo[2,3-*d*]pyrimidines: A new class of Aurora-A kinase inhibitors

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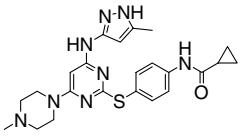
Abstract—A new class of Aurora-A inhibitors have been identified based on the 2-amino-pyrrolo[2,3-*d*]pyrimidine scaffold. Here, we describe the synthesis and SAR of this novel series. We report compounds which exhibit nanomolar activity in the Aurora-A biochemical assay and are able to inhibit tumor cell proliferation. This study culminates in compound **30**, an inhibitor with potent activity against Aurora A (IC_{50} = 0.008 μM), anti-proliferative activity against several tumor cell lines and induces polyploidy in H460 cells.

Aurora kinases have recently gained prominence as a new oncology drug target because of the critical role they play in the regulation of the cell cycle, especially in the later stages from the G2/M checkpoint through the mitotic checkpoint and late mitosis. Three Aurora kinase paralogs are expressed in mammals and at least two of the Aurora kinases (Aurora-A and -B) are commonly overexpressed in human tumors including breast, lung, colon, ovarian, and pancreatic cancers. Overexpression of Aurora-A leads to centrosome amplification and aneuploidy, and has also been shown to compromise spindle checkpoint function, allowing anaphase to occur despite continued activation of the spindle checkpoint.[1,2] Furthermore, Aurora-A has been shown to function as an oncogene.[3,4] Overexpression of Aurora-B has also been reported to produce multi-nuclearity and induce aggressive metastasis, suggesting that the overexpression of Aurora-B has multiple functions in cancer development.[5,6] Inhibition of Aurora kinase activity has been shown to induce endoreduplication and eventual cell death in cancer cells.

These findings suggest that overexpression of the Aurora kinases may play a critical role leading to chromosomal instability and inhibiting their kinase activity may have a therapeutic benefit in the treatment of cancer. Indeed, VX-680 (**1**), a potent inhibitor of Aurora-A, -B, and -C kinases, has been shown to suppress tumor growth in vivo and has now progressed into Phase 1 clinical trials.[7]



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In an effort to discover novel Aurora kinase inhibitors, our compound collection was screened against Aurora-A kinase. The pyrrolopyrimidine **2** was identified as a starting point for further optimization. This compound showed an excellent level of Aurora-A enzyme inhibition and a selectivity profile warranting further exploration. Here, we wish to report the synthesis and in vitro SAR of this new class of Aurora-A inhibitors.

Keywords: Aurora-A; 2-Amino-pyrrolo[2,3-*d*]pyrimidine; Anti-proliferative; Polyploidy.
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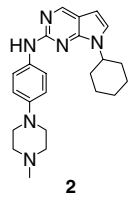
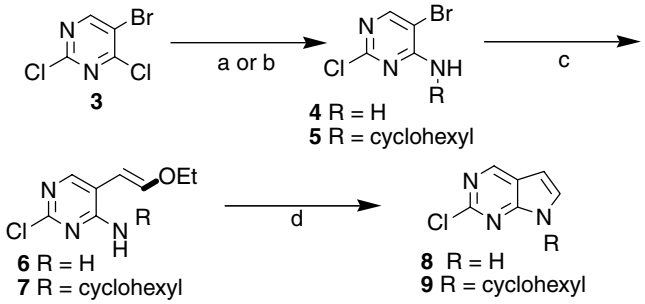


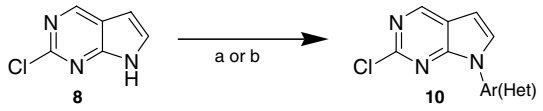
Cdk1	IC_{50} = 1.60μM
Cdk7	IC_{50} = 1.50μM
Cdk4	IC_{50} = 0.031μM
Aurora-A	IC_{50} = 0.047μM
IRK	IC_{50} = 1.00μM
c-Src	IC_{50} = 0.71μM
VEGF	IC_{50} = 0.10μM
PDGFr	IC_{50} = 0.29μM
TrkB	IC_{50} = 0.16μM

2

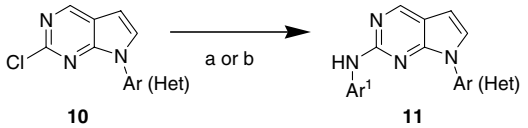
The synthesis of the pyrrolopyrimidines is described in Schemes 1–4. Using a modified procedure of a route outlined by Cheung and co-workers[8] 5-bromo-2,4-dichloropyrimidine (**3**) was treated with ammonia or 2 equiv of cyclohexylamine in THF at room temperature for 10 min. This was stirred for 12 h at room temperature or 50 °C, in the case of the cyclohexylamine reaction, to produce **4** and **5** in >95% yields. This was followed by a palladium catalyzed cross-coupling reaction with tributyl-(2-ethoxy-vinyl)-stannane and **4** or **5**, furnishing the **6** and **7** in 65% and 60% yields, respectively. Heating to 120 °C in AcOH for 2 h converted **6** and **7** to the pyrrolopyrimidines **8** and **9** in yields >85%. The use of AcOH was critical for the high conversion. The use of HCl–CH_3OH for the cyclization led to lower yields (~45%) and the isolation of several by-products.



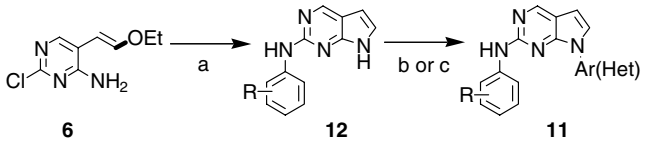
Scheme 1. Reagents and conditions: (a) NH_3, THF, rt, 12 h; (b) 2 equiv cyclohexylamine, THF, 50 °C, 12 h; (c) $Pd(PPh_3)_4$, toluene, tributyl-(2-ethoxy-vinyl)-stannane, 110 °C, 24 h; (d) AcOH, 120 °C, 2 h.



Scheme 2. Reactions and conditions: (a) $Cu(OAc)_2$, $ArB(OH)_2$, pyridine, 4 Å mol sieves, 3d, CH_2Cl_2, rt; (b) CuI, Cs_2CO_3, *N*,*N*′-dimethylcyclohexane-1,2-diamine, Ar-X (X = I, Br) or Het-X (X = I, Br), DMF, 140 °C, 24 h.



Scheme 3. Reactions and conditions: (a) $CH_3OCH_2CH_2OH$, cat HCl, 180 °C, 24 h; (b) $(t\text{-}Bu_3P)_2Pd$, K_3PO_4, DMF, 140 °C, 1 h, microwave, Ar^1NH_2.



Scheme 4. Reagents and conditions: (a) $RArNH_2$, AcOH, 120 °C, 2 h; (b) $Cu(OAc)_2$, $ArB(OH)_2$, pyridine, 4 Å mol sieves, 3d, CH_2Cl_2, rt; (c) CuI, Cs_2CO_3, *N*,*N*′-dimethylcyclohexane- 1,2-diamine, Ar-X (X = I, Br) or Het-X (X = I, Br), DMF, 140 °C, 24 h.

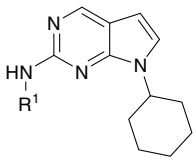
Lam–Chan[9] or Buchwald[10] coupling conditions were applied to attach the aryl groups to the N-7 atom of **8**. Yields for the coupling reaction ranged from 45% to 75% (Scheme 2). The Lam–Chan protocol was applicable for the introduction of *meta*- and *para*-substituted aryl rings at the N-7 position, however it could not be extended to ortho substituted aryl rings or heterocyclic systems. In those cases the transformation was carried out using the Buchwald procedure. The pyridine/$Cu(OAc)_2$ combination was found to be the optimal conditions for the Lam–Chan reaction while the use of Cs_2CO_3/*N*,*N*-dimethylcyclohexane-1,2-diamine at 140 °C in DMF was found to be the optimal conditions for the Buchwald conditions.

The 2-anilino-groups could be attached to **10** via two methodologies (Scheme 3). When the aniline was sufficiently nucleophilic and tolerated acidic conditions at elevated temperatures the group could be introduced thermally, otherwise, attachment was accomplished via a palladium catalyzed cross-coupling reaction. The use of the $(t\text{-}Bu_3P)_2Pd/K_3PO_4$ combination was critical for the coupling reaction. Use of other palladium reagents or bases yielded only trace amounts of the desired product or no reaction.[11] Microwave irradiation enhanced the reaction rate shortening the reaction time from 24 to 48 h for the standard thermal reaction to 1 h. Yields of **11** for the thermal and palladium reactions ranged from 25–45% to 50–80%, respectively.

The second route involved heating **6** to 120 °C for 2 h in the presence of anilines of sufficient nucleophilicity and stability in AcOH. This resulted in addition of the aniline and cyclization to **12** in one pot and in yields of 45–65%. Attachment of aryl groups or heterocycles to the N-7 atom could be done using the above-mentioned conditions (Scheme 4).

The compounds were tested for their ability to inhibit the phosphorylation of serine 10 of histone-H3 by murine Aurora-A enzyme. The compounds were also counter screened against CDK4 to establish selectivity. Table 1 outlines the SAR observed around the 2-amino portion of the pyrrolo[2,3-*d*]pyrimidine scaffold. Replacement of the 4-(4-methylpiperazin-1-yl)phenyl group led to an improvement in the overall selectivity versus CDK4, most notably compound **15**, which showed a >500-fold improvement. Modeling studies indicated that the 3-(phenyl)acetic acid of **15** was favorably

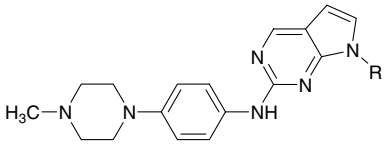
Table 1. SAR for the 2-amino modifications



Compound	R^1	IC_{50}[a] (μM)	
		Pi-Aurora ELISA	CDK4 ELISA
2	4-(4-Methylpiperazin-1-yl)phenyl	0.047	0.031
13	4-Morpholinophenyl	0.049	0.600
14	4-(2-Methoxyethyl)piperazin-1-yl	0.006	0.110
15	3-(Phenyl)acetic acid	0.0006	0.35
16	4-Phenylthiomorpholine dioxide	0.012	1.00

[a] Values are means of three experiments.

Table 2. SAR around the N-7 position



Compound	R	IC_{50}[a] (μM)	
		Pi-Aurora ELISA	CDK4 ELISA
2	Cyclohexyl	0.047	0.031
17	(Bicyclo[2.2.1]heptan-2-yl)	0.039	0.029
18	3-Methoxyphenyl	0.018	0.340
19	Pyridin-3-yl	0.013	1.4
20	3-(Methylsulfonyl)phenyl	0.009	2.0
21	2-(2,3-Dihydro-1H-indene)	0.018	2.1

[a] Values are means of three experiments.

positioned to form a salt bridge with the Arg137 of Aurora-A while the 4-(4-methylpiperazin-1-yl) group of **2** appeared to be making no observable H-bonding/charge–charge interactions with the Aurora kinase (Fig. 1). In CDK4 there is a glutamic acid situated in the same relative position and introduction of the 3-(phenyl)acetic acid moiety would potentially lead to an unfavorable charge–charge interaction resulting in a decrease in potency against CDK4.

Table 2 summarizes the SAR of the N-7 position of the pyrrolopyrimidine ring. Replacement of the cyclohexyl ring of **2** with a 3-pyridyl (**19**), 3-methylsulfonylphenyl (**20**) or dihydroindene (**21**) resulted in a significant improvement in the selectivity versus CDK4, even in the presence of the 4-(4-methylpiperazine)phenyl group.

To optimize further and determine the optimal N-7 group we carried out a more extensive exploration of this site. To get a clearer insight into the contributions the aryl/heterocyclic groups have on enzymatic potency the *para*-4-methylpiperazine ring was moved into the *meta* position of the 2-anilino ring. Although both modeling and a low resolution X-ray structure of **18** also showed the *para*-4-methylpiperazine ring not making any apparent interactions with the protein, it was believed that re-orienting the 4-methylpiperazine ring to the *meta* position (**26**) would only further minimize any possibilities of inhibitor–kinase interactions (Fig. 2).

Introduction of a wide variety of a substituted aryl and heterocyclic rings at the N-7 position was tolerated and the results from the SAR studies are summarized in Table 3. The nature and size of the substituents on the aryl ring did have an effect on the potency. Introduction of large groups (compare **26** to **27** and **28** to **29**) caused a loss in enzymatic potency while $-SO_2CH_3$ (**23**), –CN (**25**), $-OCH_3$ (**26**), and $-CONH_2$ (**28**) functionality were all tolerated.

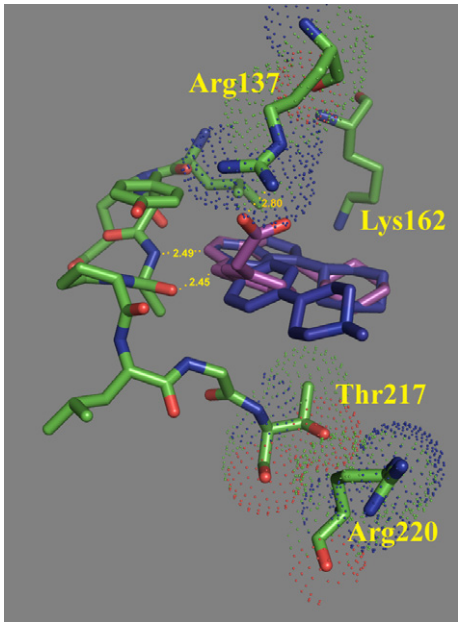


Figure 1. Model of compounds **2** and **15** docked in Aurora-A. Blue, compound **2**; magenta, compound **15**.

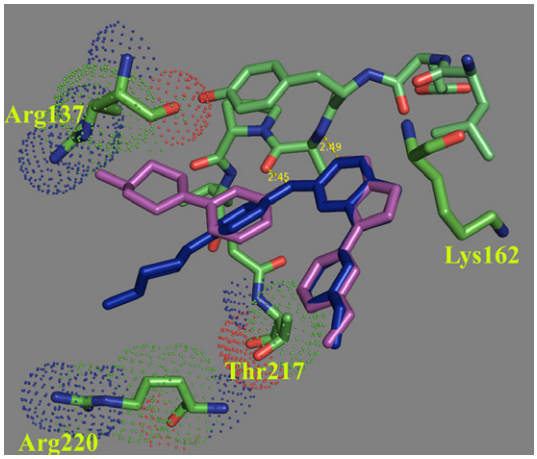
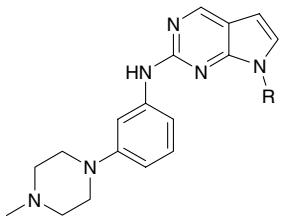


Figure 2. Model of compounds **18** and **26** docked in Aurora-A. Blue, compound **18**; magenta, compound **26**.

Table 3. SAR of N-7 position



Compound	R	Pi-Aurora ELISA IC_{50}[a] (μM)
22	Pyridin-3-yl	0.012
23	3-(Methylsulfonyl)phenyl	0.010
24	Phenyl	0.003
25	3-Benzonitrile	0.013
26	3-Methoxyphenyl	0.006
27	3-Isopropylphenyl	0.029
28	3-Benzamide	0.011
29	*N*-Cyclopentyl-3-benzamide	0.820
30	Pyridin-2-yl	0.005

[a] Values are means of three experiments.

Table 4. Proliferation assays

Compound	IC_{50}[a] (μM)		
	A2870	A375	H460
23	NT	0.44	0.86
24	0.25	0.56	0.67
26	1.20	3.67	1.58
30	0.30	0.58	0.52

NT, not tested.
[a] Data presented here are means of three independent experiments.

Four compounds (**23**, **24**, **26**, and **30**) were selected and their anti-proliferative effects evaluated in three cell lines. The results are summarized in Table 4. Compounds **24** and **30** showed the best overall activity in all three cell lines.

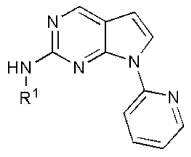
Because of its excellent enzymatic activity and better cellular potency we focused our optimization efforts on **30**. Holding the 2-pyridyl ring at N-7 constant, we concentrated our studies around the 2-amino position. From the resulting SAR studies two analogs (**31** and **32**) were identified that exhibited sub-nanomolar potency in our enzyme assay. As part of our protocol we tested **31** and **32** in human MV411 cells to assess cell penetration. Overall we found the Caco-2 assay a poor predictor of the cellular permeability characteristics of our inhibitors. So to determine if any observed loss of cellular activity against Aurora-A was potentially due to poor cellular penetration we cross screened our inhibitors in human MV411 cells. MV411s are an Flt3-driven cell line and all of the most potent pyrrolopyrimidine analogs exhibit excellent Flt3 enzyme activity with IC_{50}s < 10 nM, this allowed us to rapidly identify the best compounds to further evaluate in other cell lines. Compound **32** showed the best activity in both assays and was selected for further profiling (Tables 5–8).

Compound **32** inhibited the phosphorylation of histone-H3 Ser [10] in several tumor cell lines as measured by the Cellomics Arrayscan platform. Compound **32** also exhibited anti-proliferative activity in a variety of human tumor cell lines while showing preferential activity for cycling cells over non-cycling cells. This compound was inactive in the hERG assay (>10 μM) and only had activity against the A3 adenosine receptor (60% at 10 μM) in the 50 assay CEREP profile. Compound **32** was selective against a panel of kinases (Table 7) and had good ADME properties in human and rat (Table 8). Further, Compound **32** induced accumulation of cells with >4N DNA content and the polyploidy was accompanied by an increasing sub-G1 fraction indicative of apoptotic cell death (Fig. 3).

In summary, we have developed a series of 2-amino-pyrrolo[2,3-*d*]pyrimidine derivatives as novel Aurora-A kinase inhibitors.[12] An optimized analog **32** possessed potent in vitro activity, good kinase selectivity, and appropriate metabolic and pharmacokinetic properties. The analog was able to induce polyploidy and apoptosis, two hallmarks of Aurora kinase inhibitors.

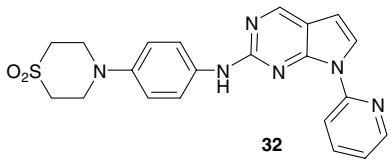
Experimental details: To measure the inhibition of aurora kinase, Histone H3 (14-494, Upstate, Charlottesville, VA) was exposed to compounds and 3 nM of purified murine Aurora-A kinase in the presence of 100 mM Hepes, pH 7.5, 1 mM DTT, 0.01% Tween 20, 10 μM ATP, and 2 mM $MgCl_2$. The reaction was terminated after 35 min and activity was determined by measuring

Table 5. Modification of R^1



Compound	R^1	Pi-Aurora ELISA IC_{50} (μM)	MV411 cell proliferation IC_{50} (μM)
30	3-(4-Methylpiperazine)phenyl	0.005	<0.013
31	3-(Phenyl)acetic acid	0.0006	0.67
32	4-Phenylthiomorpholine dioxide	0.0008	<0.013

Table 6. Assay results for compound **32**



32

Assays	IC_{50} (μM)	Cell proliferation	IC_{50} (μM)
Aurora-A	0.0008	Confluent HMECs	1.90
Flt3	<0.001	A375	0.065
Cell-based pHistoneH3 assay	0.082 (H460)	MES-SA	0.063
	0.299 (A375)	A549	0.290
	0.094 (A549)	NCI-H460	0.084
		HCT116	0.260

Table 7. Enzyme selectivity

Compound	IRK	CDK1	Src	PDGF	c-Met	Plk1	FAK
32	250	90	389	>1000	163	>1000	>1000

Selectivity ratio: IC_{50}/IC_{50} (Aurora-A).

Table 8. ADME for compound **32**

Compound	Caco-2[a] (A → B)	Caco-2[a] (B → A)	HLM[b]	RLM[b]	Cl[c]	$t_{1/2}$[d]	Rat %F
32	16	15	72	100	17	43	30

[a] app(x10-6).
[b] % Remaining at 10 min.
[c] (ml/min/kg) rat.
[d] (po, min) rat.

the phosphorylation of Ser10 of Histone H3 (9706, Cell Signaling Technologies, Danvers, MA) colorimetrically. To measure the inhibition of CDK4, retinoblastoma (Rb) protein (12-439, Upstate, Charlottesville, VA) was exposed to compounds and purified CDK4 in the presence of 100 mM Hepes, pH 7.5, 75 mM NaCl, 2 mM DTT, 30 μM ATP, and 10 mM $MgCl_2$. The reaction was terminated after 35 min and activity was determined by measuring the phosphorylation of Rb on Ser795 (#9301, Cell Signaling Technologies, Danvers, MA) colorimetrically. Two compounds were used to establish the assay VX680[7] and ZM447439[13] which showed IC_{50}s of 0.0006 and 0.039 μM, respectively.

Compounds were tested for their ability to affect the proliferation of A2780 ovarian carcinoma, A375 melanoma, MV411 human leukemia cell and NCI-H460 lung carcinoma cells grown in serum containing media. Cell proliferation was determined by the differential luminescence of cells before and after 72 h of exposure to compound when measured by CellTiterGlo (Promega Corporation, Madison, WI). Data were processed and analyzed using GraphPad Prism (version 4).

To measure the inhibition of Flt3, 20 μg/mL poly(Glu:Tyr) (4:1, Sigma P0275) was exposed to compounds and purified human Flt3 [571–933] in the presence of 100 mM Hepes, pH 7.5, 113 μM ATP, 5 mM $MgCl_2$, 1 mM DTT, and 0.01% Tween 20. The reaction was terminated after 25 min, and the extent of poly(Glu:Tyr) phosphorylation was quantitated by a fluorescent polarization competition immunoassay (Ex: 485 nm, Em: 530 nm) in the presence of an anti-phosphotyrosine antibody and a phosphotyrosine tracer (Green Tyrosine Kinase Assay Kit, PanVera P2837).

Molecular modeling studies were carried out using Accelrys Insight II and Discovery Studio software. Visualizations were done using Accelrys DS Viewer Pro 6.0 (http://www.accelrys.com) and PyMol v0.99 (http://www.pymol.org). Initial molecular models were built using the deposited Aurora-A structures 1MQ4 and 1MUO. A low resolution crystal structure (3.1 Å) of murine Aurora-A with compound **18** was later obtained that was sufficiently resolved to allow placement of the inhibitor in the active site. This structure confirmed our earlier models and was used in subsequent modeling studies.

Acknowledgments

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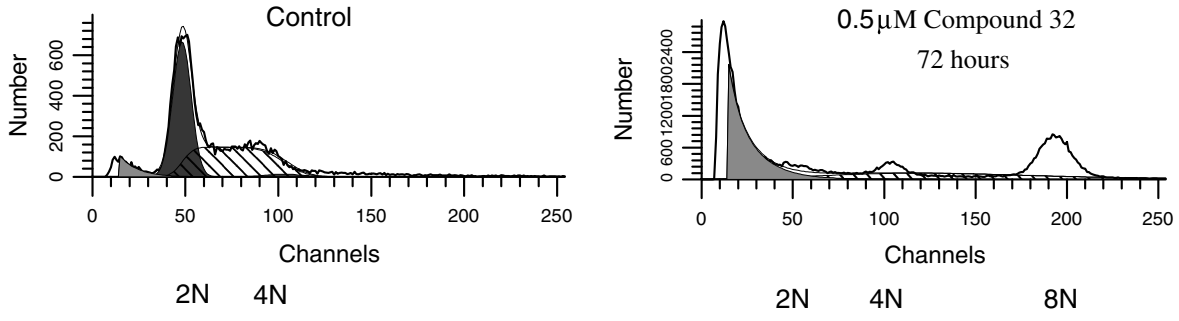

Figure 3. Flow cytometry. NCI-H460 cells were treated with compound **32** or DMSO control. The cells were harvested after 72 h and data were obtained using a BD FACSCalibur and analyzed using ModFit LT 2.0.

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