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Letter

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Azaindole-Based Inhibitors of Cdc7 Kinase: Impact of the Pre-DFG Residue, Val 195

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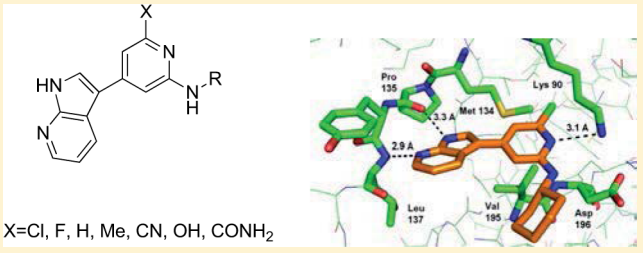
Supporting Information

ABSTRACT: To investigate the role played by the unique pre-DFG residue Val 195 of Cdc7 kinase on the potency of azaindole-chloropyridines (**1**), a series of novel analogues with various chloro replacements were synthesized and evaluated for their inhibitory activity against Cdc7. X-ray cocrystallization using a surrogate protein, GSK3β, and modeling studies confirmed the azaindole motif as the hinge binder. Weaker hydrophobic interactions with Met 134 and Val 195 by certain chloro replacements (e.g., H, methyl) led to reduced Cdc7 inhibition. Meanwhile, data from other replacements (e.g., F, O) indicated that loss of such hydrophobic interaction could be compensated by enhanced hydrogen bonding to Lys 90. Our findings not only provide an in-depth understanding of the pre-DFG residue as another viable position impacting kinase inhibition, they also expand the existing knowledge of ligand-Cdc7 binding.



KEYWORDS: *pre-DFG residue, Cdc7 kinase, azaindole*

Protein kinases are bisubstrate enzymes, possessing both a structurally well-defined ATP-binding site and a far more structurally variable substrate-binding site. Practically all medicinal chemistry efforts in the last 15 years have focused on ATP site-directed inhibitors because of the potential opportunity for the discovery of potent and selective inhibitors. While inhibitor selectivity can result from conformational plasticity of the catalytic domain, during the early stage of a kinase research program, detailed knowledge is typically limited to a far more simplistic analysis of residue variation without an advanced understanding of protein structural dynamics. The ATP site of a protein kinase is comprised of approximately 25 residues.[1] Some of these residues, such as the conserved Lys or P-loop (also termed "gly-rich loop") Gly residues, are nearly fully conserved for all kinases (95−99% conserved). At the other extreme, some residues, most notably the so-called gatekeeper, exhibit higher variation. While there is an upper limit of 20 amino acid possibilities at any one residue, no ATP site residue of any naturally occurring human protein kinase exhibits that extreme degree of variation. For example, the gatekeeper is typically found to be one of approximately eight residues[1] with Met found most often for the Ser/Thr kinases and Thr found most often for the Tyr kinases. In this paper, we describe a chemical series that allowed us to probe potency at another variable position in the ATP site: the "pre-DFG" residue. This residue immediately precedes the well-known "DFG" motif in the catalytic domain of protein kinases. Its side chain makes van der Waals (VdW) contact with many kinase inhibitor chemotypes, but its impact has previously not been well-documented.

Cdc7 (cell division cycle 7) is a serine/threonine kinase that is essential for activating the DNA replicative complex at the origins of replication.[2−5] Cdc7 functions through binding to Dbf4 or Drf1, a regulatory subunit.[3,5,6] The Cdc7/Dbf4 complex promotes S-phase entry by phosphorylating the minichromosome maintenance complex 2−7 (MCM2−7) protein, preferably MCM2, at multiple sites (e.g., Ser 40 and Ser 53).[7−9] Cdc7/Dbf4 has been found to be overexpressed in various tumor cell lines as well as in human cancers including breast, colon, lung, leukemia, etc.[10,11] Because Cdc7 overexpression promotes proliferation and survival of certain tumor cells, knockdown of Cdc7 by siRNA has led to p53-independent apoptosis in cancer cell lines.[12,13] Tumor cells with the often impaired S-phase checkpoints progress to mitosis without DNA repair. Meanwhile, upon Cdc7 depletion, normal cells are able to arrest in the G1 phase of the cell cycle and resume normal divisions once an initiation process is restored.[12] This differential regulation in tumor versus normal cells presents a potential opportunity to develop novel agents targeting Cdc7 inhibition as anticancer therapeutics.

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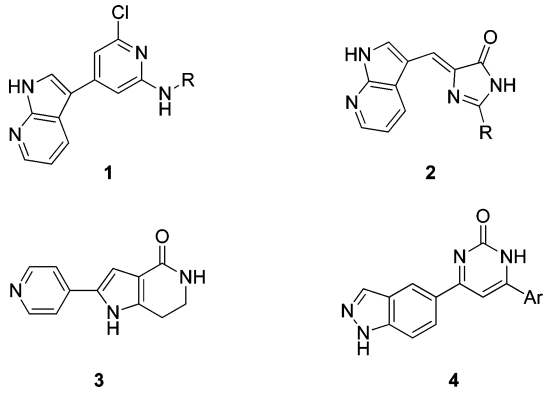


In the past few years, several organizations have disclosed small-molecule Cdc7 inhibitors.[14−17,22] Among them, Nerviano Medical Sciences has published comprehensive studies covering pyrrolopyridine- (e.g., **2**[18]), pyrrolopyridinone- (e.g., **3**[19,20]), and pyrrolocarboxamide[21]-based Cdc7 inhibitors. Some pyrrolopyridinones, such as PHA-767491 (**3**, 2-pyridin-4-yl-1,5,6,7-tetrahydro-pyrrolo[3,2,*c*]pyridine-4-one), have been shown to inactivate replication origins, induce apoptosis in cancer cells, and inhibit tumor growth in animal models.

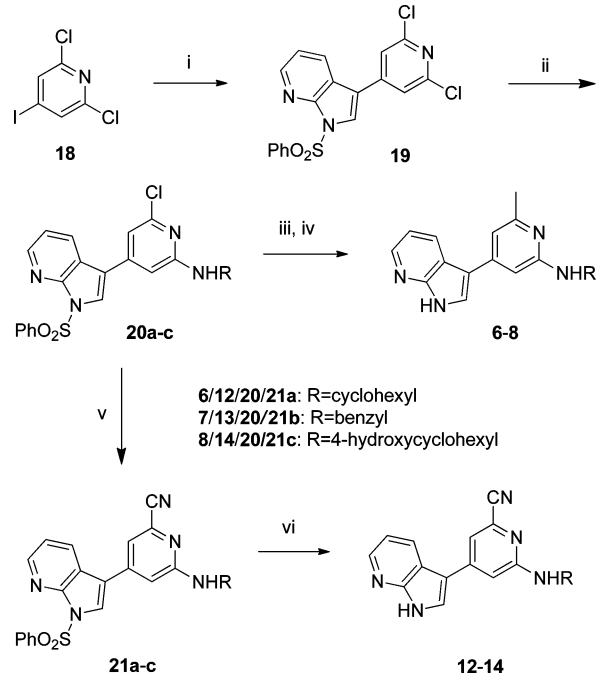
We recently disclosed a novel class of molecules with an azaindole-pyrimidinone scaffold as potent inhibitors for Cdc7.[22] Further development in medicinal chemistry led to inhibitors containing an azaindole-chloropyridine core (**1**). However, without a lactam carbonyl as seen on **2**−**4**[16] and our earlier inhibitors, it is not obvious whether **1** binds to the hinge region of the ATP-binding site with its pyridine or the azaindole moiety. Meanwhile, the unique pre-DFG residue of Cdc7, Val 195, which precedes the DFG motif of Asp 196, Phe 197, and Gly 198, warrants an elaborated SAR study to map the region since this area has not been explored by earlier publications. This letter will first disclose the X-ray cocrystallography and modeling work that defines the binding mode of the chloropyridines (**1**). It will then focus on the analogue studies that illustrate exploitation of the pre-DFG residue in kinase inhibitor discovery.

The synthesis of **6**−**8** and **12**−**14** is shown in Scheme 1. The commercially available pyridine derivative **18** was subjected to a Suzuki reaction to yield the bis-chloro intermediate **19**. The following S_NAr reaction with an amine replaced one of the two chloro groups. The initial Suzuki couplings between **20** and methylboronic acid only led to desulfonylated starting materials. However, reactions in the presence of $AlMe_3$ and a Pd catalyst replaced the targeted chloro on **20** with a methyl. Subsequent removal of the phenylsulfonyl protecting group under basic conditions completed the synthesis of **6**−**8**. Compound **20** could also react with $Zn(CN)_2$ catalyzed by Pd_2dba_3 to give **21**. The standard basic deprotection resulted in analogues **12**−**15**.[23] A similar deprotection of **20a** afforded **16**.

To synthesize final products **9**−**11** (Scheme 2), the difluoropyridyl compound **23** was first obtained from 2,6-difluoro-4-iodopyridine (**22**),[24] under typical Suzuki reaction conditions. Substituting one of the fluoro groups with a suitable amine at 160 °C in a microwave reactor followed by deprotection of the azaindole moiety gave **9** and **10**. The amine addition could also be accomplished on a deprotected difluoro intermediate **24**, leading to **11**.

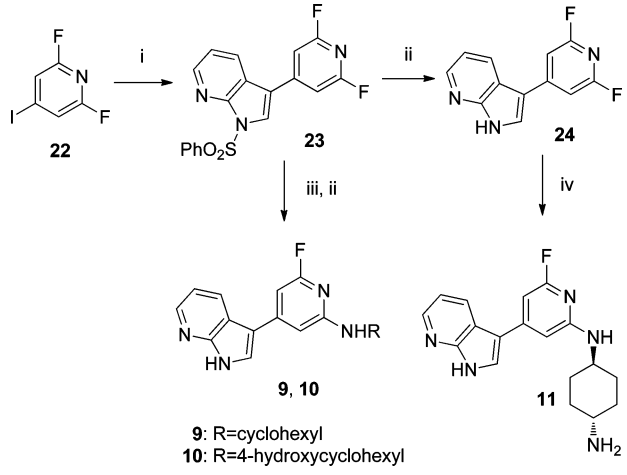
All of the inhibitors synthesized were evaluated by a biochemical assay to measure their inhibitory activity (reported as K_i values; see the Supporting Information for details).

Scheme 1[a]



[a]Reagents and conditions: (i) 1-(Phenylsulfonyl)-3-(pinacoboranyl)-pyrrolopyridine, $Pd(PPh_3)_2Cl_2$, Na_2CO_3, DME/EtOH/H_2O, 80 °C, quan. (ii) Amines, microwave, 160−170 °C, 30−135 min, 41−56%. (iii) $AlMe_3$, $Pd(PPh_3)_4$, DMF, 80 °C. (iv) NaOH, dioxane, 100 °C, 16−25% (2 steps). (v) $Zn(CN)_2$, Zn, Pd_2dba_3, dppf, DMA, 120 °C, 1.5 h or microwave, 170 °C, 30 min, 44−67%. (vi) NaOH, dioxane, 100 °C, 1.7−30%.

Scheme 2[a]



[a]Reagents and conditions: (i) $Pd(PPh_3)_2Cl_2$, Na_2CO_3, 1-(phenylsulfonyl)-3-(pinacoboranyl)-pyrrolopyridine, EtOH/DME/H_2O, 80 °C, 63%. (ii) NaOH, dioxane, 50 °C. (iii) Amine, EtOH, microwave, 150 °C. (iv) *trans*-Cyclohexane-1,4-diamine, EtOH, microwave, 170 °C, 40%.

Replacing the chloro of inhibitor class **1** may have a profound impact on potency. As demonstrated in Table 1, the fluoro analogues (**9**−**11**) are generally as potent as their chloro counterparts. However, the hydrogen (**5**), methyl (**6**−**8**), cyano (**12**−**14**), and carboxamide (**15**) substitutions led to varied degrees of erosion on the Cdc7 inhibitory activity.

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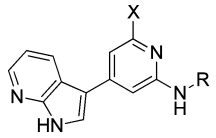
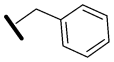
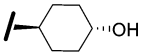
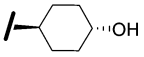
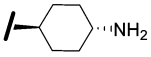
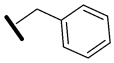
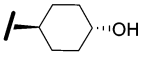
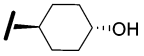
Table 1. Effect of Cl Replacements



	X	R	Ki (nM)[a]	Ki ratio (X/Cl)[b]
16	Cl		0.90	1
17[c]	OH		0.28	0.31
5	H		8.7	9.7
6	Me		13	14
7	Me		15	60
8	Me		27	54
9	F		3.2	3.6
10	F		1.7	3.4
11	F		3.4	2.4
12	CN		7.7	8.5
13	CN		1.4	5.6
14	CN		5.5	11
15	$CONH_2$		67	134

[a]Average of two measurements. [b]K_i ratio: each example/corresponding Cl analogue at X. [c]See the synthesis in ref 22.

Understanding of ligand binding to Cdc7 kinase is based on published structural studies[16,18,19,25] of compounds **2**−**4** using surrogates. Attempts to crystallize Cdc7 have not been successful to date, so structural information derives from either crystallographic studies of ligand-bound surrogate kinases, such as PIM or GSK3β kinases, or ligand docking into homology models of Cdc7 kinase. The high architectural conservation of the kinase ATP site allows these surrogate and modeled structures to be useful for SAR interpretation, even when sequence identity of the entire catalytic domain is low. The emerging structural understanding[16,18,19,25] indicates that there are two dominant binding features within the ATP site: (1) a ligand heterocycle, such as pyrrolopyridine (azaindole), pyridine, or indazole, serves as a hinge-binding unit, and (2) a directly connected lactam functionality, such as imidazolone, pyridone, or pyrimidone, that interacts with the conserved lysine residue. In Cdc7 kinase, these regions are created by Pro 135 and Leu 137 within the hinge and Lys 90 with its nearby Met 134, the "gatekeeper" residue, and Val 195, the "pre-DFG" residue. The pre-DFG residue is the residue that immediately precedes the well-known kinase DFG motif and has its side chain form part of the binding surface around the conserved Lys residue. Val 195 is of high interest as the pre-DFG residue of Cdc7 as it is proposed to influence ligand potency, vide infra.

Structural consideration of the binding mode of compounds of type **1** indicates that they contain two heterocycles (pyridine and azaindole) known to serve as hinge binders for Cdc7 kinase but do not have a lactam unit to interact with the conserved Lys. Therefore, the binding mode was uncertain. A crystal structure of compound **16** bound to GSK3β kinase,[26] shown in Figure 1, provided unambiguous evidence that the azaindole

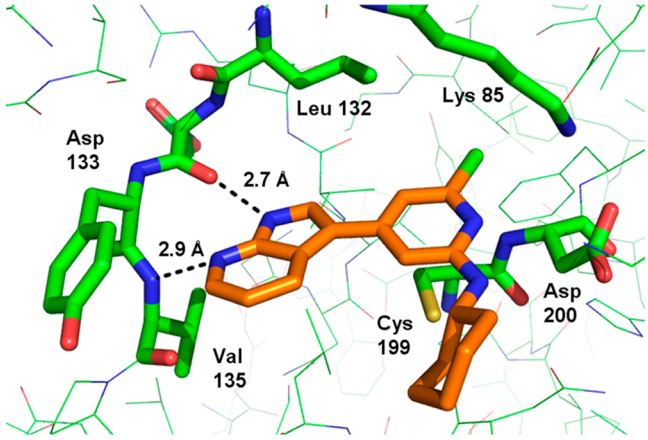


Figure 1. Protein crystal structure of compound **16** in GSK3β kinase. Hinge hydrogen bonds to Asp 133 and Val 135 are shown in black dashed lines. The gatekeeper and pre-DFG residues of GSK3β kinase are Leu 132 and Cys 199, respectively.

unit served as the hinge-binding unit and that the pyridine pointed toward the conserved Lys in this surrogate kinase. Also, the twist of the pyridine ring relative to the azaindole is seen to be flatter, at about 10°, than what would have been expected for a biaryl system with a fused ring. The potency of compound against GSK3β kinase was sufficient (K_i = 58 nM)[27] that the binding mode is likely to be analogous in Cdc7. Furthermore, the presence of an ortho-chloro group would likely be sterically confounding if the pyridyl unit were taken to be the hinge-binding unit. Therefore, further modeling work in Cdc7 kinase proceeded with the general binding mode assumed as observed in GSK3β kinase.

The conserved Lys is well-precedented to donate an H-bond to ligand acceptors in many kinase inhibitors. The surrounding hydrophobic environment is created by the side chains of the gatekeeper and pre-DFG residues. A model of compound **16** in Cdc7 kinase is shown in Figure 2. The chloro substituent is observed to make van der Waals contact with the gatekeeper Met 134 and Val 195. The twist of the pyridine ring is observed to be relatively low (23°) to maximize the van der Waals interaction between the pyridine ring and the Val 195 and is supported by the low twist angle observed in the X-ray structure with GSK3β. While Met is a common gatekeeper residue found in many kinases, Val is an uncommon pre-DFG residue. Eighty-three percent of all kinases have their pre-DFG residues as Gly, Ala, Ser, Thr, or Cys.[29] Only 4% of the kinome (20 kinases; listed in the Supporting Information) have the sterically larger and more hydrophobic Val as the pre-DFG residue. The potency of Cdc7 inhibitors that vary structurally in this conserved Lys region will likely be driven by some combination of (a) the degree and arrangement of the hydrophobic interaction with the gatekeeper Met 134 and pre-DFG Val 195 leading to a restricted volume relative to other kinases with smaller pre-DFG residues and (b) the degree of polar interaction with the conserved Lys side chain. Our

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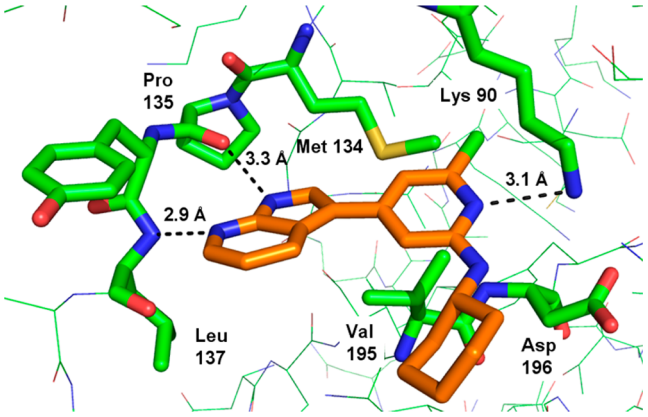

Figure 2. Model of chloropyridine compound **16** in Cdc7 kinase.[28] Hinge hydrogen bonds to Pro 135 and Leu 137 are shown. The gatekeeper, Met 134, and pre-DFG residue, Val 195, are highlighted to show their proximity to the conserved Lys 90. The chlorine atom of the inhibitor is in VdW contact with the terminal atoms of Met 134 and Val 195.

rationalization of the SAR in Table 1 invokes both binding features. Replacement of the chloro with hydrogen (**5**) or methyl (**6**) led to about one log potency loss, relative to **16**. A simple tabulation of calculated LogP for benzene analogues and the corresponding inhibitors, shown in Table 2, shows that

Table 2. ClogP Values[a]

substrate	ClogP	inhibitor	ClogP
Ph-H	2.14	**5**	4.12
Ph-F	2.28	**9**	4.39
Ph-Me	2.64	**6**	4.62
Ph-Cl	2.86	**16**	4.96
Ph-CN	1.57	**12**	4.06
Ph-OH	1.47	**17**	2.45
Ph-$CONH_2$	0.65		

[a]Calculated using BioByte.

because of the lower LogP, both of these analogues would be expected to have diminished hydrophobic interaction in this region of the active site. Replacement with the two-atom cyano group, **12**–**14**, or three-atom carboxamide group, **15**, led to potency losses of 5–11- and 134-fold, respectively, from a possible net combination of factors: loss of hydrophobic interaction (Table 2), steric clash with a constricted hydrophobic environment, and/or suboptimal hydrogen bonding between the substituent and the Lys 90. In the cases of the one-atom fluoro analogues, compounds **9**–**11**, or oxo analogue, pyridinone **17**, inhibition strengths typically within 3× of the corresponding chloro analogues were observed. While both fluoro and oxo would be expected to exhibit less hydrophobic interaction with gatekeeper and pre-DFG side chains, their atomic positions would be expected to be approximately 3 Å from the ε-amino group of Lys 90. Therefore, both are able to compensate with enhanced H-bond ability with Lys 90, resulting in little net potency difference.

In summary, the binding mode of the chloropyridines (**1**) as potent Cdc7 inhibitors was clarified by a cocrystal X-ray structure of **16** in a surrogate protein, GSK3β, confirming the azaindole moiety of the molecule as the hinge-binding motif. Meanwhile, dissecting the impact of the chloro on potency called for a thorough understanding of the structural biology around the region occupied by the gatekeeper, Met 134, and the pre-DFG residue, Val 195, of the Cdc7 kinase. By synthesizing a series of novel analogues with various chloro replacements, we established the interplay among hydrogen bonding, hydrophobic interaction, and steric influence in this particular ATP-binding region. The reduced hydrophobic interaction of the chloro replacements (i.e., H, Me, and CN) with Met 134 and Val 195 led to compounds (**5**, 7, and **12**–**14**) with deteriorated Cdc7 inhibition. However, the loss of such hydrophobic interaction could be compensated by enhanced hydrogen bonding to Lys 90 as demonstrated by analogues such as **9**–**11** and **17**. The findings in this paper not only provide an in-depth understanding of the pre-DFG residue as another viable position impacting the kinase inhibition, they also expand the existing knowledge of ligand-Cdc7 binding.

■ ASSOCIATED CONTENT

Supporting Information

List of kinases having valine as the pre-DFG residue, X-ray crystallography method and statistics, GSK3β protein purification protocol, assay protocols, and synthetic procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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