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Synthesis of aminopyrazole analogs and their evaluation as CDK inhibitors for cancer therapy

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Abstract

We synthesized a library of aminopyrazole analogs to systematically explore the hydrophobic pocket adjacent to the hinge region and the solvent exposed region of cyclin dependent kinases. Structure-activity relationship studies identified an optimal substitution in the hydrophobic pocket and analog **24** as a potent and selective CDK2/5 inhibitor.

Graphical Abstract

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Supplementary data

Supplementary data associated with this article can be found, in the online version.

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Uncontrolled cell proliferation is a hallmark of all cancers. Members of cyclin-dependent kinase (CDK) family of proteins are involved in the regulation of cell cycle and transcription.^{1, 2} Thus pharmaceutical companies and academic laboratories have targeted CDK for cancer therapy. A number of CDK inhibitors built on different core structures (Figure 1) have been developed and a handful of CDK inhibitors have advanced through clinical trials and obtained approval or orphan drug status from the FDA (Flavopiridol - pan CDK, CLL; Palbociclib - CDK4/6, breast cancer; Ribociclib - CDK4/6, breast cancer; and Abemaciclib - CDK4/6, breast cancer).³⁻⁶ Additionally, drug leads such as AT7519 with the aminopyrazole core are in clinical trials for cancer therapy.⁷ The aminopyrazole core interacts with the hinge region residues of kinases by forming a triad of hydrogen bonds (Figure 2A).⁸ We recently showed that an aminopyrazole analog (CP668863 a.k.a. 20-223) developed as a CDK5 inhibitor by Pfizer for neurodegenerative diseases inhibited tumor growth in a xenograft mouse model.⁹ In vitro and cellular assays showed that 20-223 was a CDK2/5 inhibitor.⁹

CDK2 is involved in cell cycle regulation, and either over-expression of CDK2 or inactivation of its endogenous inhibitors (CIP/KIP family of proteins) is linked to various cancers.¹⁰ Consistently, inhibition of CDK2 by small molecule result in growth inhibition and apoptosis in a panel of cancer cell lines.¹¹ CDK5 is an atypical cyclin-dependent kinase, best known for its role in the central nervous system and regulates development, axon elongation, and neuronal migration.¹² Unlike other CDKs, which are activated by cyclins, CDK5 is activated by regulatory proteins p35 and p39. Recently, CDK5 was found to possess non-neuronal functions, which include apoptosis, senescence, angiogenesis, insulin secretion, wound healing, and adhesion/migration, many of which are the hallmarks of tumorigenesis and thus an attractive target for therapeutic intervention against cancer.^{9, 13-16} Herein, we report the synthesis and screening of aminopyrazole analogs as CDK2/5 inhibitors.

The nitrogen atoms of the aminopyrazole core interact with the hinge region residues of CDK2 and the substituent at the 5-position (R^1) of the aminopyrazole is embedded in a shallow hydrophobic pocket (P1) whereas the substituent at the R^2 position is solvent exposed.⁸ The conformation of analog **16** docked into CDK5 (PDB: 1UNL) (Figure 2B), overlaid well with the **16**:CDK2 co-crystal structure (PDB: 1VYW). This indicates that aminopyrazole analogs will likely target both CDK2 and CDK5.

We constructed a 40-member library wherein the size of the R^1 substitution was systematically increased to explore the hydrophobic pocket, and we used aryl substituents with and without oxygen atoms at the R^2 position to explore the solvent exposed region of CDKs (Figure 2C). We hypothesized that the R^2 substituents with the oxygen atoms will not be subjected to desolvation penalty and therefore will have improved potency for CDKs.¹⁷

The R^1 substituted aminopyrazoles were either commercially available or synthesized in two steps from the corresponding R^1 -esters (Scheme 1).⁸ The internal nitrogen atom on the pyrazole core was more nucleophilic and was therefore Boc-protected.⁸ To access analogs **36-40**, the Boc protected phenyl-fused aminopyrazole was synthesized in a single step by a copper mediated condensation of *t*-butyl carbazate and 2-iodobenzonitrile (Scheme 1).¹⁸ The

Boc-protected aminopyrazoles were condensed with various R² substituted acids or acid chlorides, and the removal of the Boc protecting group on the resulting amides yielded the aminopyrazole library (1-40) (Figure 2C).^{17, 19-26}

The aminopyrazole library was screened against a small panel of CDKs, *viz.*, CDK2/cyclin E, CDK4/cyclin D1, CDK5/p35, CDK6/cyclin D1, CDK7/cyclin H and CDK9/cyclin T1 at 100 nM in cell-free kinase assays (Figure 3A). Irrespective of the CDK, the activities of the compounds increased when the substituent at R¹ was larger than isopropyl or smaller than cyclopentyl (across the row – Figure 3C). However, we saw no clear preference at the R² position (Figure 3A). This probably indicates that the substituents at the R² position are not truly surface exposed. We set a threshold of >90% inhibition, which led to the identification of 14 aminopyrazole analogs (blue box, Figure 3) as inhibitors selective for CDK2 and CDK5 in our library. Five (**21-25**) of the nine analogs identified as the top hits had a cyclobutyl substituent at the R¹ position, but no such clustering was observed for the R² position.

Using caspase 3/7 activation assay as a readout, we evaluated these fourteen analogs for their ability to induce apoptosis.²⁷ MiaPaCa2 cells were subjected to 14 aminopyrazole analogs (10 μM) for 12h, and a caspase activity assay was performed. This secondary screen identified three aminopyrazole analogs (**21**, **24**, and **25**) that induced greater than two-fold increase in caspase activation (Figure 4). Interestingly, all three analogs (**21**, **24**, and **25**) had a cyclobutyl substituent at the R¹ position and a hydrophobic substituent at the R² position.

Next, we subjected a panel of Pancreatic Ductal Adenocarcinoma (PDAC) cell lines to the top three compounds (**21**, **24** and **25**) to identify the most potent inhibitor of cell growth. AsPC1, BxPC3, MiaPaCa2, SUIT3, and S2-013 cells were treated with **21**, **24**, or **25**, for 72h and then analyzed for inhibition of cell proliferation using the PrestoBlue assay.^{28, 16} AT7519, and Roscovitine, known CDK inhibitors with potencies in the low-nM to μM range were used as control compounds to benchmark the potency of our compounds against inhibitors in clinical trials. This screen revealed that analog **24** had nanomolar potency in all five cell lines and was more potent than AT7519 and Roscovitine (Table 1).

To determine in vitro selectivity of **24**, we performed a cell-free dose-response study with a small panel of kinases. Analog **24** effectively inhibited CDK2/cyclin E (24 nM), and CDK5/p35 (23 nM) with low-nM potencies. Analog **24** was >35-fold selective for CDK2/5 over CDK9. We also screened **24** against a panel of other kinases inhibited by analogs with the aminopyrazole core (Aurora A,²⁹ Aurora B, DLK/MAP3K12, ITK,³⁰ JAK2,³¹ JNK3,³² LRRK2,³³ P38a/MAPK14,³² PAK1,³⁴ TRKA). Remarkably the IC₅₀ value for the entire panel was > 10 μM, indicating that analog **24** was selective for CDK2, and CDK5 (Table 2). Analog **24** was also inactive against unrelated kinases IKKβ and BTK. ML-120B (an IKKβ inhibitor) and Ibrutinib (a BTK inhibitor) were used as positive controls.

In conclusion, a focused 40 member library of aminopyrazole based compounds was synthesized. Structure activity relationship studies revealed that a cyclobutyl substitution at the R¹ position was optimal for activity and no clear selectivity for substitution at the R² position was observed. Follow up screens with hits, which included, an apoptosis assay, and

growth inhibition studies led to the identification of analog **24** with sub- μ M potency. Moreover, profiling **24** against a small panel of other kinases showed excellent selectivity for CDK2 and CDK5 with low-nM potency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A series of aminopyrazole based CDK2/5 were designed and synthesized.
- A screening funnel of cell-free and Caspase 3/7 assay identified three analogs.
- Growth inhibition studies in a panel of pancreatic cancer cell lines revealed analog 24 a lead compound.
- In vitro selectivity assay against a panel of kinases showed excellent selectivity for CDK2 and CDK5.

Figure1.
Structures of CDK inhibitors.

Figure 2. Design of aminopyrazole library. (A) Aminopyrazole core forms a triad of potential hydrogen bonds with the hinge region residues of CDK. (B) Aminopyrazole analog (**16**) docked into the ATP binding pocket of CDK5 (PDB code 1UNL). (C) R¹ and R² substituents in the 40-member library.

Figure 3. Cell-free CDK profiling with 40 aminopyrazole library at 100 nM. >90% inhibition of CDK2 and CDK5 by aminopyrazole analogs (blue box).

Figure 4.
Fold-change in Caspase 3/7 activation of top 14 aminopyrazole analogs at 10 μ M in MiaPaCa2 cells for 12h.

Scheme 1.

Synthesis of aminopyrazole analogs. Reagents and conditions (i) CH_3CN , KOt -amyl, THF (40%); (ii) N_2H_4 , EtOH, Reflux (82%); (iii) $(BOC)_2O$, KOH, DCM:H₂O (1:1) (85-92%); (iv) R^2 (acid chloride), DCM, 0°C (40-80%) or R^2 (Acid), EDCI, NEt_3 , DCM (50-75%); (v) CF_3CO_2H , 0°C, DCM (75-90%). (vi) *t*-butyl carbazate, CuI, K_2CO_3 , DMSO, RT (45%).

Table 1.

IC₅₀ values from 72h growth inhibition (GI) assays with **21**, **24**, **25** and clinically used CDK inhibitors, AT7519 and Roscovitine.

Cell line	IC ₅₀ (nM) ± SEM				
	21	24	25	AT7519	Roscovitine
AsPC1	1163 ± 105	315 ± 17	> 2000	533 ± 33	17767 ± 1284
BxPC3	> 2000	924 ± 62	> 2000	640 ± 55	15284 ± 633
MiaPaCa-2	861 ± 49	247 ± 26	1559 ± 62	411 ± 37	18214 ± 1148
SUIT2	> 2000	209 ± 16	> 2000	557 ± 148	14801 ± 2515
S2-013	875 ± 120	192 ± 20	1468 ± 264	2770 ± 558	21700 ± 3500

Table 2.Kinase profiling of **24** against a panel of kinases.

Kinases	IC ₅₀ (nM) of 24	Kinases	IC ₅₀ (nM) of 24
CDK2/cyclin E	24 ± 3	DLK/MAP3K12	>10000
CDK5/p35	23 ± 2	ITK	>10000
CDK9/	911 ± 148	JNK3	>10000
BTK ^a	>10000	LRRK2	>10000
IKKβ ^b	>10000	P38a/MAPK14	>10000
Aurora A	>10000	PAK1	>10000
Aurora B	>10000	TRKA	>10000

^aIbrutinib = 0.21 ± 0.01 nM,^bML 120B = 465 ± 74 nM