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Versatile templates for the development of novel kinase inhibitors: Discovery of novel CDK inhibitors

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Abstract—A series of four bicyclic cores were prepared and evaluated as cyclin-dependent kinase-2 (CDK2) inhibitors. From the in-vitro and cell-based analysis, the pyrazolo[1,5-*a*]pyrimidine core (represented by **9**) emerged as the superior core for further elaboration in the identification of novel CDK2 inhibitors.

The cyclin-dependent kinases (CDKs) are a family of serine/threonine kinases that function as critical regulators of the mammalian cell cycle which integrates extracellular signaling, DNA synthesis, and mitosis.[1] Dysregulation of cell cycle control is a hallmark of all human cancers and is frequently associated with aberrant activation/regulation of cyclin-dependent kinases (CDKs).[2] While coordinated CDK2/CDK1 activity is required for appropriate regulation of S-phase entry (DNA synthesis), suppression of apoptosis in late S-phase, S-phase exit and entry into mitosis, it has been illustrated that inhibition of CDK2/CDK1 in tumors provokes cell cycle arrest and apoptosis.[3] Hence, inhibition of the essential, rate-limiting activities of CDK2 and CDK1 represents an attractive therapeutic strategy for oncology indications.[4]

Several CDK inhibitors are currently under clinical evaluation including flavopiridol (**1**),[5] roscovitine (**2**),[6] BMS 387032 (**3**),[7] R547 (**4**),[8] and PD0332991 (**5**) (Fig. 1).[9] However, opportunities exist to identify and develop additional novel CDK inhibitors that may possess superior biological profiles to current candidates. One considerable challenge that exists in this area is the identification of novel core structures for the development of selective kinase inhibitors. Toward this end, the targetting of ATP competitive inhibitors of the CDKs has emerged as the mainstay of this area with several classes of compounds having been developed.[10]

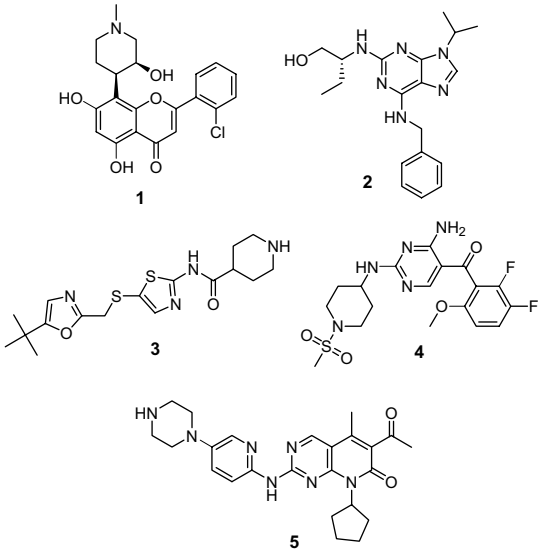

Figure 1. CDK inhibitors currently under clinical evaluation.

Keywords: CDK2; Kinase inhibitors; Pyrazolo[1,5-*a*]pyrimidine.
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Herein, we report our initial efforts exploring the utility of several bicyclic cores toward the development of novel ATP-competitive kinase inhibitors of the CDKs.

In the early course of our CDK program, two lead classes of compounds emerged from our in-house screening: the pyrazolo[1,5-*a*]pyrimidine **6**[11] and the imidazo[1,2-*a*]pyrazine **7** both of which possessed submicromolar potency in a cyclin A/CDK2 kinase biochemical assay[12] (Fig. 2).

Owing to the structural similarities of **6** with the purine CDK2 inhibitor roscovitine (**2**) (Fig. 1), initial C3 bromide substitution of **8** yielded compound **9** which demonstrated over 10-fold improvement of potency in the biochemical assay[12] (Fig. 3). Having achieved reasonable potency in compound **9**, efforts were undertaken to explore the potential utility of other bicyclic cores outside those represented by **6** and **7**. Toward this end, we targetted two additional cores besides the pyrazolo-[1,5-*a*]pyrimidine derivative **9** and the imidazo[1,2-*a*]pyrazine **10** to include: pyrazolo[1,5-*a*]pyridine **11**; and imidazo[1,2-*a*]pyridine **12** (Fig. 3). Each of these compounds was prepared bearing identical substitution and subsequent biological evaluation would allow for the identification of the best CDK bicyclic scaffold.

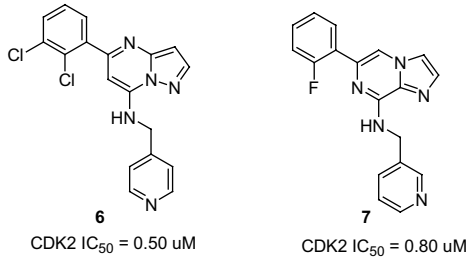


Figure 2. Pyrazolo[1,5-*a*]pyrimidine **6** and imidazo[1,2-*a*]pyrazine **7** lead structures from library screening.

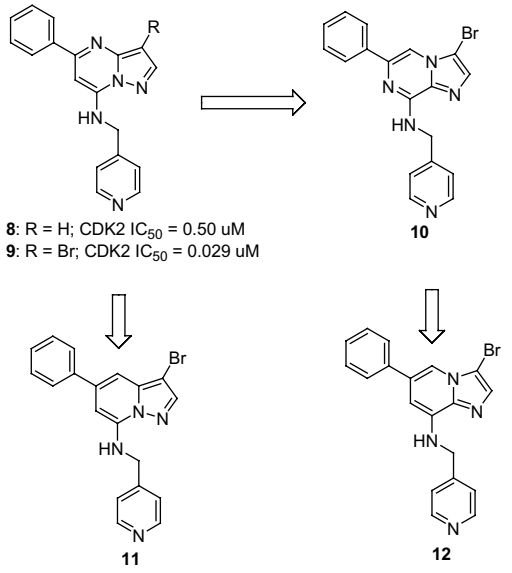
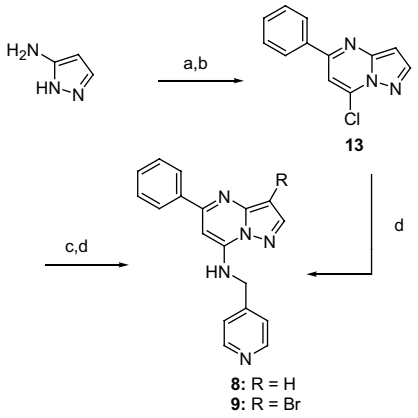
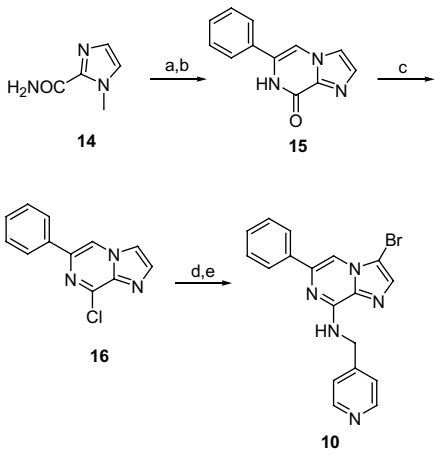


Figure 3. Four bicyclic cores of interest as potential CDK inhibitors.

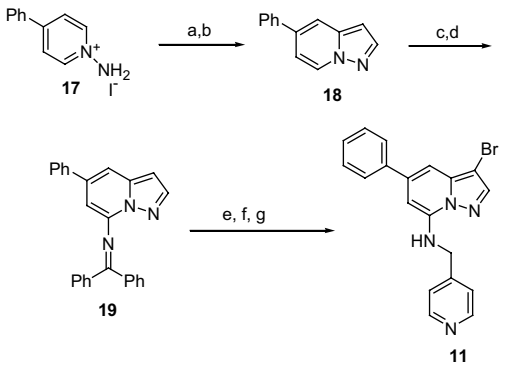
The preparation of the individual core molecules **8**–**12** is shown in Schemes 1–4.



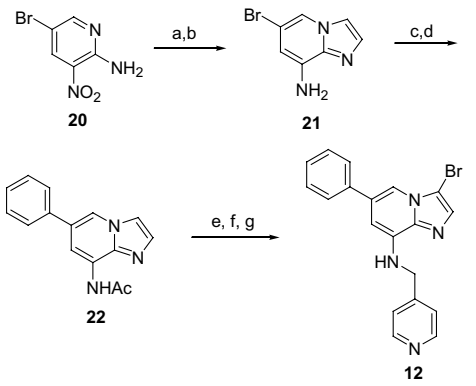
Scheme 1. Reagents and condition: (a) ethyl benzoylacetate, AcOH, 100 °C; (b) $POCl_3$, pyridine, DMAP; (c) NBS, CH_3CN; (d) 4-pyridylmethylamine, DIPEA, dioxane.



Scheme 2. Reagents and condition: (a) α-bromomethylphenyl ketone, CH_3CN; (b) imidazole, heat; (c) $POCl_3$, pyridine, DMAP; (d) NBS, CH_3CN; (e) 4-pyridylmethylamine, DIPEA, dioxane.



Scheme 3. Reagents and conditions: (a) ethyl propiolate, K_2CO_3, air; (b) H_2SO_4, heat; (c) *n*-BuLi, diiodoethane; (d) Ph_2CNH, $Pd(OAc)_2$, BINAP, Cs_2CO_3, toluene; (e) NBS, CH_3CN; (f) NH_2OH, NaOAc; (g) 4-pyridinecarboxaldehyde, $ZnCl_2$ then $NaBH_3CN$.



Scheme 4. Reagents: (a) $SnCl_2 \cdot H_2O$, EtOH; (b) $BrCH_2CHO$, K_2CO_3; (c) $PhB(OH)_2$, $Pd(PPh_3)_4$, K_3PO_4, DME/H_2O; (d) AcCl, pyridine; (e) NBS, CH_3CN; (f) aq HCl, EtOH; (g) 4-pyridinecarboxaldehyde, $ZnCl_2$ then $NaBH_3CN$.

Preparation of the pyrazolo[1,5-*a*]pyrimidine derivatives **8** and **9** was achieved by treatment of 3-aminopyrazole with ethyl benzoylacetate under acid conditions followed by chlorination under standard conditions to afford compound **13** (Scheme 1).[13] Regioselective bromination followed by displacement with 4-pyridylmethylamine afforded the title compound **9** while treatment with the amine directly on **13** afforded compound **8**.

Preparation of the imidazo[1,2-*a*]pyrazine analog **10** was carried out in an analogous fashion to the protocol depicted in Scheme 1. Treatment of 1-methyl-1*H*-imidazole-2-carboxamide[14] **14** with α-bromomethylphenyl ketone followed by dealkylation of the resultant quaternary salt with imidazole afforded compound **15** (Scheme 2).[14] Conversion to chloride **16** followed by regioselective bromination and amine displacement yielded title compound **10**.

Preparation of the pyrazolo[1,5-*a*]pyridine analog **11** began with cyclization of the known 1-aminopyridinium adduct **17**[15] with ethyl propiolate under basic conditions followed by acid-catalyzed decarboxylation to afford cycloadduct **18** (Scheme 3).[16] Regioselective iodination[17] followed by Pd-catalyzed amination afforded imine **19**. Regioselective bromination, imine deprotection, followed by $ZnCl_2$-promoted reductive amination afforded the title compound **11**.

Preparation of the imidazo[1,2-*a*]pyridine adduct **12** began with treatment of nitro adduct **20** under reductive conditions[18] followed by cyclization with in-situ generated bromoacetaldehyde to afford **21**.[19] Suzuki coupling with phenyl boronic acid followed by acetylation afforded **22**. Regioselective bromination, deprotection, and reductive amination afforded the title compound **12**.

The targets described above were assayed in a biochemical assay for the inhibition of cyclin A/CDK2.[12] Analogs that demonstrated reasonable potency in the biochemical assay were advanced into a thymidine incorporation assay[20] which was used to measure the ability of the compounds to inhibit asynchronously growing A2780 ovarian carcinoma cells. The data generated for compounds **9**–**12** and flavopiridol are summarized in Table 1.

Table 1. Cyclin A/CDK2 assay[12] and thymidine incorporation assay[20] for analogs **9**–**12** and flavopiridol

Compound	Cyclin A/CDK2 IC_{50} (μM)[a]	Thymidine incorporation IC_{50} (μM)[a,b]
9	0.029	0.6
10	0.44	2.0
11	4.5	—
12	0.70	—
Flavopiridol	0.012	—

[a] All IC_{50} values are means of at least two determinations.
[b] Assay conditions listed in Ref. 20.

In Table 1, incorporation of a C3 bromide into imidazo[1,2-*a*]pyrazine series (**7**–**10**) showed a clear improvement in in-vitro potency (twofold) but to a lesser extent as was observed in the pyrazolo[1,5-*a*]pyrimidine series (represented by **9**). Modification of the bicyclic core had a much more dramatic effect on the CDK2 in-vitro potency as displayed in Table 1. Deletion of the N6 nitrogen in the imidazo[1,2-*a*]pyrazine adduct **10** yielded imidazo[1,2-*a*]pyridine **12** which led to a twofold loss in in-vitro potency. More noticeably, the deletion of the N4 nitrogen of pyrazolo[1,5-*a*]pyrimidine **9** afforded pyrazolo[1,5-*a*]pyridine **11** which suffered a 100-fold loss in in-vitro potency. The great disparity in potency based upon the nature of the bicyclic core is suggestive that the placement of N atoms in cores such as **9** and **10** is imperative. This functionality may play a role in mediating the H-bond donor/acceptor capability of the core or possibly pick up additional interactions with the protein. Our in-house X-ray data[21] for compound **9** bound to CDK2 elucidated several key polar interactions between the 7-NH and Leu83 backbone carbonyl and the pyrazole N and Leu83 backbone NH in a purine-like binding mode (Fig. 4). Recent X-ray data of related pyrazolo[1,5-*a*]pyrimidine analogs[11] are consistent with these results while additional computational and X-ray structural data rationalizing the

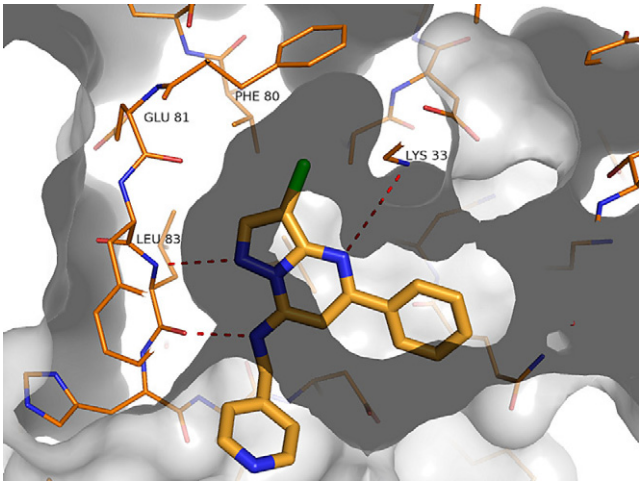


Figure 4. X-ray structure of **9** bound to CDK2.[21] Red dotted lines represent key polar interactions.

dramatic potency differences between the core structures **9**–**12** will be reported in due course.[22] The two most potent analogs (**9** and **10**) were further evaluated for their ability to alter uptake and incorporation of radioactively labeled thymidine by living cells. In line with the observed in-vitro potency trends in Table 1, pyrazolo-[1,5-*a*]pyrimidine adduct **9** possessed superior activity in the thymidine incorporation assay with an IC_{50} = 0.6 μM versus the comparable imidazo[1,2-*a*]pyrazine adduct **10**. As summarized in Table 1, pyrazolo[1,5-*a*]pyrimidine adduct **9** displayed comparable in-vitro potency in the cyclin A/CDK2 assay to the known CDK inhibitor flavopiridol (**1**) shown in Figure 1.

In summary, four bicyclic cores were designed and prepared bearing identical functionality based upon early screening hits. Based upon both in-vitro and cell-based data, the pyrazolo[1,5-*a*]pyrimidine core (represented by **9**) emerged from these efforts as the preferred bicyclic motif for our CDK2 program. Further optimization of the pyrazolo[1,5-*a*]pyrimidine series as CDK2 inhibitors appears in the accompanying paper.[23]

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12. *Cyclin A/CDK2 kinase assay*. Recombinant baculoviruses were purified from Sf9 cells engineered to express cyclin A and CDK2. Cyclin A/CDK2 enzyme was diluted to a final concentration of 50 μg/mL in kinase buffer containing 50 mM Tris, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mM sodium orthovanadate. For each kinase reaction, 1 μg of enzyme and 20 μL of 2 μM substrate solution (a biotinylated peptide derived from Histone H1; Amersham, UK) were mixed and combined with 10 μL of diluted compound. The reaction was started by addition of 50 μL of 2 μM ATP and 0.1 μCi of 33P-ATP (Amersham, UK). Kinase reactions ran for 1 h at room temperature and were stopped by the addition of 0.1% Triton X-100, 1 mM ATP, 5 mM EDTA, and 5 mg/mL streptavidin-coated SPA beads (Amersham, UK). SPA beads were captured using a 96-well GF/B filter plate (Packard/Perkin Elmer Life Sciences) and a Filtermate universal harvester (Packard/Perkin-Elmer Life Sciences.) Beads were washed twice with 2 M NaCl and twice with 2 M NaCl with 1% phosphoric acid. Signal was then assayed using a TopCount 96-well liquid scintillation counter (Packard/Perkin-Elmer Life Sciences). Dose–response curves were generated from duplicate 8 point serial dilutions of inhibitory compounds. IC_{50} values were derived by nonlinear regression analysis.
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20. *Thymidine uptake growth inhibition assay*. The thymidine incorporation assay was used to measure inhibition of asynchronously growing A2780 ovarian carcinoma cells exposed to test compound. These cells were maintained in DMEM (Cellgro) plus 10% fetal bovine serum (HyClone) and passaged twice a week by detaching the monolayer with Trypsin-EDTA (Gibco). One hundred microliters of A2780 cells (5000 cells/well) was added per well to a 96-well Cytostar T plate (Amersham, UK) and incubated for 16–24 h at 37 °C. Compounds were serially diluted in complete media plus 2% ^{14}C-labeled thymidine (Amersham, UK). Media were removed from Cytostar T plate, 200 μL of compound dilution was added in quadruplicate and incubated for 24 h at 37 °C. Accumulated incorporation of radiolabel was assayed using scintillation proximity and measured on TopCount (Packard/Perkin-Elmer Life Sciences). Percentage inhibitions, relative to vehicle controls, were calculated and plotted on log-linear plots to allow derivation of IC_{50} values.
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