Letters

Indenopyrazoles as Novel Cyclin Dependent Kinase (CDK) Inhibitors

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Introduction. A fundamental process in biology is the division of cells mediated by the cell cycle. This process ensures the controlled production of subsequent generations of cells with defined biological function. Overexpression of the tumor promoting components or the loss of tumor suppressing products can lead to unregulated cellular proliferation and the generation of tumors.[1]

Cyclin dependent kinases (cdks) play a key role in regulating the cell cycle machinery. Each kinase associates with a specific regulatory cyclin and together make up the active catalytic moiety. The coordinated activity of these kinases guides cells through the replication process and ensures the vitality of each subsequent generation.[2]

An increasing body of evidence has shown a link between tumor development and cdk-related malfunctions.[3] This evidence has led to an intense search for small molecule inhibitors of the cdk family as an approach to cancer chemotherapy.[4] Several disclosures have discussed small molecules with cdk inhibitory activity.[5] To date, only two compounds (flavopiridol and UCN-01) have entered into clinical trials as cancer therapeutics based on this cdk inhibition mechanism.[6] We disclose here a new structural class of potent and selective cdk inhibitors, which are active against transformed cell lines and show in vivo activity in a human xenograft mouse model.

High-throughput screening (HTS) of our compound collection against cdk4/D1 gave several interesting compounds including A3915 (Figure 1). Subsequent screening of this hit against other cdks as well as other relevant kinases showed the compound to be selective for the cdk family. We began a medicinal chemistry effort around A3915 in an attempt to improve its potency against the cdk family while maintaining selectivity against our panel of kinases.

Chemistry. The synthesis of these compounds is shown in Scheme 1. The starting point for the synthesis was dimethyl 3-nitrophthalate readily converted to the acetamide **1** in two high-yielding steps. We chose the acetamide as an aniline protecting group due to its ability to withstand the harsh basic conditions of the triketone formation reaction. It could then be removed under acidic conditions to provide aniline **4** for further
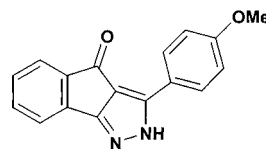

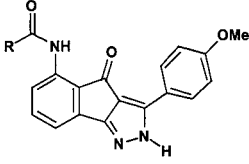| k4/D1 IC$_{50}$ (μM)	k2/E IC$_{50}$ (μM)	PKA IC$_{50}$ (μM)	PKC IC$_{50}$ (μM)	c-Abl IC$_{50}$ (μM)
45	26	239	>725	>725

Figure 1. High-throughput lead A3915.

analogue generation. The original procedure for preparing tricarbonyl compounds such as **2** used sodium methoxide in refluxing toluene.[7] We found that NaH in DMF at 90 °C gave much better results for this transformation. The tricarbonyl intermediate **2** was then treated with hydrazine to give the indenopyrazole **3** as a single regioisomer. The acetamide group provides an additional benefit during the treatment with hydrazine to form the pyrazole. An intramolecular hydrogen bond between the acetamide NH and the adjacent carbonyl affects this group's reactivity, and subsequent attack by hydrazine occurs at the other carbonyl group, leading to the one isomeric indenopyrazole shown in Scheme 1. This initial assignment was by 2D NMR experiments and subsequently confirmed by X-ray crystallography.[8] Removal of the acetamide group was accomplished with concentrated HCl in refluxing methanol.

Results and Discussion. The aniline intermediate **4** was useful in preparing additional analogues in this series,[9] and the SAR of selected analogues is presented in Table 1.[10] There is a good correlation between the amide substituent's size and its activity against both

Scheme 1^a
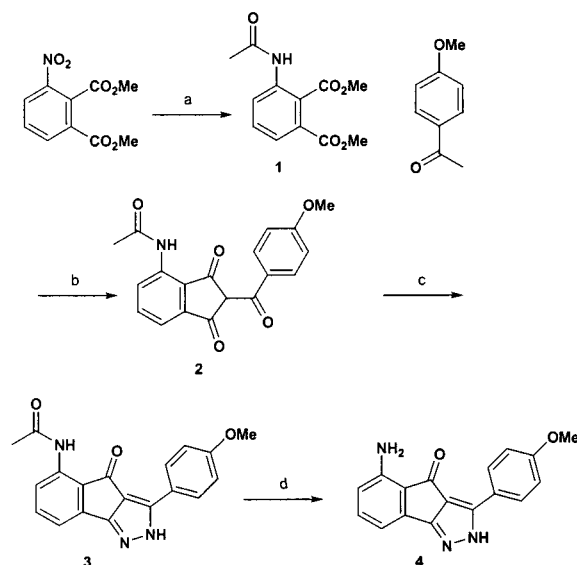
^{a} Reagents and conditions: (a) 1. H$_2$, Pd/C, 2. Ac$_2$O, pyridine, 25 °C, 79%; (b) 2 equiv of NaH, DMF, 90 °C, 30%; (c) hydrazine, cat. *p*-TsOH, EtOH, reflux, 50%; (d) conc. HCl, MeOH, reflux, 96%.

Table 1. Enzymatic and Cellular Activity for Selected Compounds

cmpd	R	k4/D1 IC$_{50}$ (μM)^a	k2/E IC$_{50}$ (μM)^a	HCT 116 IC$_{50}$ (μM)^b	AG1523 IC$_{50}$ (μM)^b
5	H	0.2	0.08	0.69	>63
3	Me	0.45	0.27	2.7	
6	*i*-Pr	35	1.4	2.9	>28
7	*t*-Bu	45	2.1	7.5	
8	4-NH$_2$C$_6$H$_4$CH$_2$	0.48	0.038	0.4	>24
9	Ph	>250	41	N/M^c	N/M^c

^{a} Values correspond to $n = 2$. For assay conditions, see ref 13.
^{b} Values correspond to $n = 2$. For assay conditions, see ref 14. ^{c} N/M = not measured.

Table 2. In Vivo Antitumor Activity for Compound **5**

cmpd	dose ip (mg/kg/day)^a	tumor wt (mg $\pm$ SE)	tumor growth inhibition (%)^b	no. survived^c
control		1069 $\pm$ 107		
$\pm$-flavopiridol	7.5	538 $\pm$ 76	50	5/8
mitomycin C	1	455 $\pm$ 40	58	8/8
5	30	608 $\pm$ 54	43	8/8
5	10	871 $\pm$ 52	19	8/8
5	3	1171 $\pm$ 40	0	8/8

^{a} Dosing schedule: Q1DX14. ^{b} For assay conditions, see ref 15.
^{c} Refers to acute drug toxicity not long-term survival.

cdk4 and cdk2. Small aliphatic groups provide compounds with good cdk inhibitory activity (**3**, **5**). Branching at the α-carbon (**6**, **7**) as well as a phenyl substituent (**9**) reduced activity. By adding a methylene bridge we were able to expand the size and character of the amide substituents while maintaining good activity (**8**). In general, the compounds are more active against cdk2 compared to cdk4. One example (**8**) is 10-fold selective, favoring cdk2. All analogues maintain good selectivity against other kinases from our selectivity panel.[11] The cellular activity of these compounds was also routinely good against a transformed colon cell line (HCT116). Here too the cellular activity parallels the cdk inhibitory activity. We also tested the compounds for their cytotoxic effects in a normal fibroblast cell line (AG1523). The compounds have minimal influence on the mortality of this cell line, indicating a promising therapeutic window for this series against transformed cell lines.

We chose compound **5** for further in vivo evaluation and dosed this compound in a human xenograft mouse model. The compound was given ip once a day for 14 days, and the tumor size was evaluated at the end of the experiment based on weight. As shown in Table 2, there is a dose dependent response to the tumor growth inhibition. At 30 mg/kg, compound **5** had an effect on tumor growth approaching that of flavopiridol.[12] In addition, all eight animals survived the dosing regimen and appeared healthy and normal at the end of the experiment.

Conclusion. We disclosed a new structural class of cyclin dependent kinase inhibitors. These compounds are selective for the cdk related serine/threonine kinase family and are active in cell culture against a transformed colon cell line (HCT116). In addition, these compounds demonstrate in vivo activity by reducing tumor growth in a human xenograft mouse model in a dose-dependent manner. Additional studies are underway to improve the potency of these compounds and to optimize the compound's physical properties to facilitate additional in vivo experiments.

Supporting Information Available: Complete experimental details for **1**, **2**, **3**, and **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Selective acylation of the aniline amine in the presence of the free pyrazole NH was accomplished using an excess of the desired acid chloride in dioxane with aqueous saturated NaHCO$_3$ as the base at 45 °C for 1 h. Standard workup and recrystallization from ethanol gave the desired products in good yield. The formamide group of compound **5** was introduced by heating aniline **4** in neat formic acid at 100 °C for 1 h. After the solvent was evaporated and the residue was recrystallized from ethanol, compound **5** was obtained in 80% yield.

(10) Spectroscopic data for selected compounds: **5**: mp 280 °C; ^{1}H NMR (300 MHz, DMSO) 13.6 (s, 1 H), 10.35 (s, 1 H), 8.5 (s, 1 H), 8.2 (d, 8 Hz, 1 H), 8.1 (d, 8 Hz, 2 H), 7.5 (t, 8 Hz, 1 H), 7.2 (d, 8 Hz, 1 H), 7.05 (d, 8 Hz, 2 H), 3.8 (s, 3 H). CIMS *m/e* Calcd for $C_{18}H_{14}N_3O_3$: 320.1035. Found: 320.1040. Anal. Calcd for $C_{18}H_{13}N_3O_3$: C, 67.71; H, 4.10; N, 13.16. Found: C, 67.55; H, 4.22; N, 13.00. **6**: mp 288 °C; ^{1}H NMR (300 MHz, DMSO) 13.6 (s, 1 H), 10.2 (s, 1 H), 8.2 (d, 8 Hz, 1 H), 8.1 (d, 8 Hz, 2 H), 7.5 (t, 8 Hz, 1 H), 7.2 (d, 8 HZ, 1 H), 7.05 (d, 8 Hz, 2 H), 3.8 (s, 3 H), 2.6 (m, 1 H), 1.5 (d, 15 Hz, 6 H). CIMS *m/e* Calcd for $C_{21}H_{20}N_3O_3$: 362.1505. Found: 362.1535. **8**: mp 283 °C; ^{1}H NMR (300 MHz, DMSO) 13.6 (s, 1 H), 10.2 (s, 1 H), 8.2 (d, 8 Hz, 1 H), 8.1 (d, 8 Hz, 2 H), 7.5 (t, 8 Hz, 1 H), 7.2 (m, 3 H), 7.05 (d, 8 Hz, 2 H), 6.5 (d, 9 Hz, 2 H), 5.0 (s, 2 H), 3.8 (s, 3 H), 3.2 (s, 2 H). CIMS *m/e* Calcd for $C_{25}H_{21}N_4O_3$: 425.1614. Found: 425.1643.

(11) Values were measured for these compounds against PKA, PKC, and c-Abl. All had IC_{50} values greater than 250 μM.

(12) The solubility of compound **5** limited us from dosing at concentrations higher than those presented in Table 2.

(13) The in vitro assays employ cell lysates from insect cells expressing either of the kinases and subsequently their corresponding regulatory units. The cdk/cyclin lysate is combined in a microtitre-type plate along with a kinase compatible buffer, ^{32}P-labeled ATP at a concentration of 50 mM, a GST-Rb fusion protein, and the test compound at varying concentrations. The kinase reaction is allowed to proceeded with the radiolabled ATP, and then it is effectively stopped by the addition of a large excess of EDTA and unlabeled ATP. The GST-Rb labeled protein is sequestered on a GSH-Sepharose bead suspension, washed, and resuspended in scintillant, and the ^{32}P activity was detected in a scintillation counter. The compound concentration which inhibits 50% of the kinase activity was calculated for each compound.

(14) We examined the effect of these compounds on cultured HCT116 cells and determined their effect on cell-cycle progression by the colorimetric cytotoxcity test using sulforhodamine B (Skehan et al. *J. Natl. Cancer Inst.* **1990**, *82*, 1107−12). HCT116 cells are cultured in the presence of test compounds at increasing concentrations. At selected time points, groups of cells are fixed with trichloroacetic acid and stained with sulforhodamine B (SRB). Unbound dye was removed by washing, and protein-bound dye was extracted for determination of optical density.

(15) HCT116 human colon carcinoma cells were injected subcutaneously at a concentration of 1×10^7 cells/animal into the inguinal region of female nude mice. The test drugs were administered intraperitoneally once a day, every day, for 14 days beginning seven days following cell injection. On day 15 postdrug administration, tumors were removed and weighed and the tumor growth inhibition index (%) was determined according to the following formula: tumor growth inhibition index (%) = (1- mean net tumor weight of experimental group/mean net tumor weight of control group) × 100.