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Identification of Selective Inhibitors of Cyclin Dependent Kinase 4

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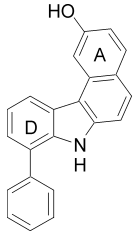
Abstract—A new structural type of kinase inhibitor, containing a benzocarbazole nucleus, has been identified. Members of the series are selective for inhibition of the cyclin dependent kinase family of enzymes. Although the cdks are highly homologous, representatives of the series showed intra-cdk selectivities, especially for cdk4. SAR studies elucidated the important features of the molecules for inhibition.

The eucaryotic cell cycle is regulated by the coordinated activity of a family of cyclin dependent kinases (cdks).[1] The enzymes, consisting of a kinase and a regulatory cyclin subunit, become active at specific phases of the cycle. For example, cdk1/cyclin B becomes active late in the G2 phase of the cell cycle, while cdk2/cyclin E is important late in the G1 phase. The complexes of cdk4 and cdk6 with D-type cyclins are especially interesting as they control cell cycle entry. Activation of cdk4 results in a specific phosphorylation of the retinoblastoma protein (pRb) which is critical to the release of transcription factors of the E2F family.[2] The Rb kinase pathway is frequently deregulated in transformed cells.[3] For example, pRb and p16 (a specific inhibitor of cdk4/6) have been identified as the products of tumor suppressor genes while cyclin D1 is a proto-oncogene product. These factors make specific inhibitors of cdk4 highly interesting targets for pharmacological intervention in cancer.[4]

A high-throughput screening campaign using cdk4/cyclin D1 complex and a GST-tagged, truncated pRb succeeded in identifying the carbazole **1** as an inhibitor. Kinetic analysis suggested that **1** is a competitive inhibitor with respect to ATP. The apparent selectivity for cdk4/D1 over other related cdks prompted us to initiate a chemistry effort to explore the activity of this class of molecules.



Cdk4/D1 IC_{50} = 3.6 μM
Cdk2/E IC_{50} = 97 μM
Cdk1/B IC_{50} = 95 μM

1

The literature route to **1** consists of the reaction of 2,7-dihydroxynaphthalene with 2-hydrazinobiphenyl in the presence of sodium bisulfite. We found that the slightly longer route consisting of a Fischer indole synthesis followed by aromatization of the B ring (Scheme 1) resulted in higher yields and was more convenient for analoging purposes.[5]

Several findings emerge from the data in Table 1. The presence of a hydrogen bond donor in the A ring appears to be important. Removal (example **9**) or methylation (example **4**) results in analogues devoid of activity. Movement of the hydroxy moiety to the 1- or

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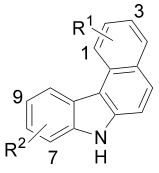
the 3-position on the A ring results in less potent inhibitors, although example **7** in which the hydroxyl group is at the 4-position is surprisingly active in this regard. Similar investigations indicate that the phenyl group on the D ring contributes less to the overall binding affinity than the phenol and is optimally disposed when attached to the 7-position.

To further define the nature of the A ring interaction, we prepared the aza analogues and their corresponding *N*-oxides. The route uses a stilbene photocyclization to assemble the ring system.[6] As shown in Table 2, these analogues are all less potent than the reference compound **13**. The fact that the *N*-oxides cannot substitute for the phenol strongly suggests that a hydrogen bond donor is critical and the lack of activity of **4** is likely not the result of an unfavorable steric interaction.

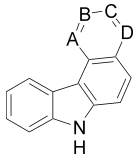
Scheme 1. Synthesis of positional isomers.

Table 1. Inhibition of Cdk4/D1



Compd	R^1	R^2	Cdk4/D1 IC_{50} (μM)
1	2-OH	7-Ph	3.6
4	2-MeO	7-Ph	>309
5	1-OH	7-Ph	19.5
6	3-OH	7-Ph	29
7	4-OH	7-Ph	5.8
8	2-NH_2	7-Ph	27.3
9	H	7-Ph	>341
10	2-OH	8-Ph	22
11	2-OH	9-Ph	37.5
12	2-OH	10-Ph	83
13	2-OH	H	17.4

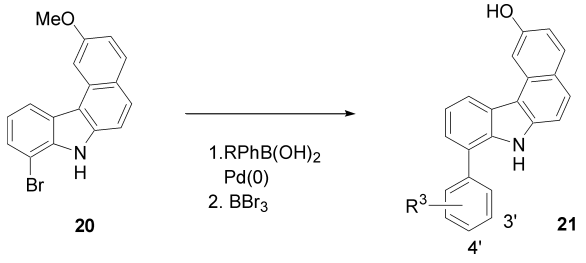
Table 2. Inhibition of Cdk4/D1 by aza analogues



Compd	A	B	C	D	Cdk4/D1 IC_{50} (μM)
14	N	CH	CH	CH	442
15	CH	N	CH	CH	159
16	CH	CH	N	CH	30
17	CH	CH	CH	N	197
18	CH	NO	CH	CH	605
19	CH	CH	NO	CH	59

The SAR of the D ring phenyl may be conveniently explored via Suzuki couplings on the bromide **20** (Scheme 2). As the data in Table 3 indicate, substitution is well tolerated on the phenyl ring. Significantly, example **22** is 10-fold more soluble than **1**. Example **22** also retains the selectivity within the CDK family displayed by **1** (cdk1/B IC_{50} = 74.5 μM). In addition, IC_{50}'s for inhibition of other kinases including c-abl, PKA, and PKC are at least two orders of magnitude higher than that observed against cdk4.

The properties of four of the analogues in in vitro cell assays are shown in Table 3. Exposure of colon carcinoma HCT116 to these agents causes cell growth inhibition accompanied by cell death with IC_{50}'s in the 10 μM range as judged by an SRB assay. The Skut1A/1B pair are Rb^- and Rb^+, respectively. The prediction is that a fully selective cdk4 inhibitor would effect only the Rb^+ cells since Rb is the only known substrate of



Scheme 2. Synthesis of substituted phenyl analogues.

Table 3. Cellular activity of selected cdk4/D1 inhibitors

Compd	R^3	Cdk4/D1 IC_{50} (μM)	HCT116 IC_{50} (μM)	Skut 1A IC_{50} (μM)	Skut 1B IC_{50} (μM)
1	H	3.6	10.2	10.7	2.8
22	3′-NH_2	2.3	9.6	11.6	4.1
23	4′-OH	1.9	2.2	5.6	2.3
24	4′-Cl	9.9	51	36.0	10.2

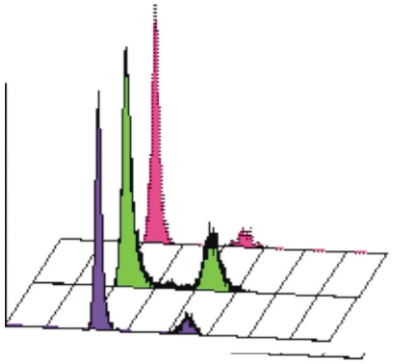


Figure 1. FACS analysis of compound **1** on HS27 fibroblasts. Primary human fibroblasts (HS27) were arrested using serum starvation and released with 10% serum in the presence or absence of compound **1**. Profile 1 (blue) serum starved cells. Profile 2 (green): 24 h after the addition of 10% serum to serum starved cells. Profile 3 (red): 24 h after the addition of 10% serum and compound **1** to serum starved cells.

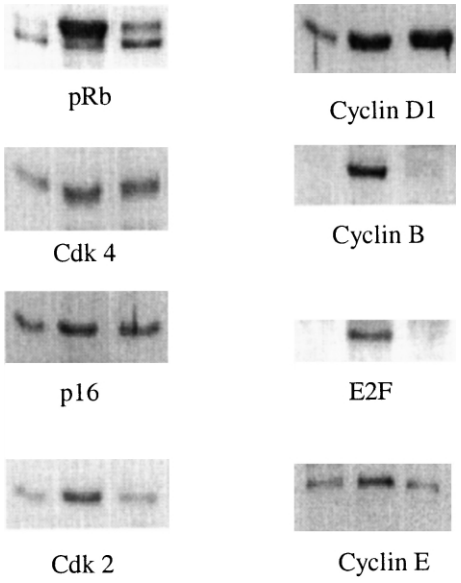


Figure 2. Western-blot analysis of fibroblast G1 release. Equal protein from cellular extracts from the G1 release experiment described in Figure 1 were tested for the expression of selected cell cycle related proteins using Western-blot analysis. Lane 1: Serum starved cells. Lane 2: 24 h after the addition of 10% serum to serum starved cells. Lane 3: 24 h after the addition of 10% serum and compound **1** to serum starved cells.

the enzyme. Compounds **1** and **22** distinguish between the Skut 1A and Skut 1B cells, consistent with their enzymatic selectivity.

The behavior of **1** in cells was examined in more detail. Figure 1 shows a FACS analysis of serum starved cells arrested in G0/G1 and treated with **1**.[7] The compound prevents serum induced cell cycle re-entry, consistent with a cdk4 blockade and failure to progress to S phase. BrDU incorporation analysis (data not shown) also demonstrated a loss of S phase cells.

Western-blot analysis of fibroblasts treated with **1** is consistent with a cdk4 inhibition mechanism.[8] There is a clear increase in dephosphorylated pRb without detectable alteration in cyclin D1 or cdk4 levels. The endogenous protein inhibitor p16 is also unaffected by exposure to **1** (Figure 2). Enzymes and transcription factors that are downstream of cdk4 are present in diminished quantities. Thus, cdk2, cyclin B, cyclin E, and E2F-1 are expressed at levels similar to serum starved cells.

In conclusion, we have found a series of carbazole analogues with interesting intra-cdk selectivity profiles. These activities translate into a consistent profile in whole cell assays. The dim relationship of these compounds to known classes of kinase inhibitors suggests that elucidation of the structural basis of cdk4 inhibition will provide useful clues for the design of more potent and selective analogues.

References and Notes

1. Morgan, D. O. *Annu. Rev. Cell Dev. Biol.* **1997**, *13*, 261.
2. Dyson, S. *Genes Dev.* **1998**, *12*, 2245.
3. Pines, J. *Sem. Cell. Biol.* **1994**, *5*, 399.
4. Sielecki, T. M.; Boylan, J. F.; Benfield, P. A.; Trainor, G. L. *J. Med. Chem.* **2000**, *43*, 1.
5. Katritzky, A. R.; Wang, Z. *J. Heterocycl. Chem.* **1988**, *25*, 671.
6. DeSilva, O.; Snieckus, V. *Synthesis* **1971**, *5*, 254.
7. Cell cycle analysis. Primary human fibroblast cells were obtained from ATCC (HS27) and maintained in MEM media supplemented with glutamine and 10% heat-inactivated FCS. Cells were G1 arrested using serum starvation and released with the addition of 10% FCS. Cells were harvested 24 h following serum release. Cell cycle analysis was performed using the standard Propidium Iodine (PI) assay (in *Current Protocols in Cytometry*; Robinson, J. P., Ed.; John Wiley & Sons: New York, 1998; Vol. 1).
8. Protein extracts were tested for the expression of selected cell cycle related proteins using Western-blot analysis. For Western blots, cellular extracts were diluted in sample buffer (2% SDS, 10% glycerol, 10 mm β-mercaptoethanol, and 60 mM Tris–HCl, pH 6.8) and heated for 5 min at 95 °C. Samples were stored at −70 °C. Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the indicated primary antibody using standard techniques. The level of protein expression was visualized using the ABC Vectastain kit (Vector Labs, Burlingame, CA) and Chemiluminesence (NEN, Boston, MA). Antibodies were obtained from the following sources: p16 (Calbiochem, San Diego, CA), cyclin E, cdk2, cdk4, E2F-1, cyclin B, cyclin D1 (Santa Cruz, CA), pRb (Pharmingen, San Diego, CA), and actin (Amersham, Piscataway, NJ).