Synthesis, Structure−Activity Relationship, and Biological Studies of Indolocarbazoles as Potent Cyclin D1-CDK4 Inhibitors

Guoxin Zhu,* Scott E. Conner, Xun Zhou, Chuan Shih, Tiechao Li, Bryan D. Anderson, Harold B. Brooks, Robert Morris Campbell, Eileen Considine, Jack A. Dempsey, Margaret M. Faul, Cathy Ogg, Bharvin Patel, Richard M. Schultz, Charles D. Spencer, Beverly Teicher, and Scott A. Watkins

Lilly Research Laboratories, A Division of Eli Lilly & Company, Lilly Corporate Center, Indianapolis, Indiana 46285

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Abstract: Novel substituted indolocarbazoles were synthesized, and their kinase inhibitory capability was evaluated in vitro. 6-Substituted indolocarbazoles **4** were found to be potent and selective D1/CDK4 inhibitors. **4d** and **4h** exhibited potent and ATP-competitive D1/CDK4 activities with IC_{50} values of 76 and 42 nM, respectively. Both compounds had high selectivity against the other kinases. These D1/CDK4 inhibitors inhibited tumor cell growth, arrested tumor cells at the G1 phase, and inhibited pRb phosphorylation.

Introduction. Cancer has been recognized as a disease of uncontrolled cell proliferation. Consequently, the genes that regulate cell proliferation have been the targets of cancer chemotheraphy. The cell cycle is a series of highly regulated processes that result in the duplication of a cell.[1] Much evidence suggests that cell cycle progression is controlled by the sequential activation of a series of cyclin-dependent kinases (CDKs). This family of serine/threonine protein kinases is composed of a catalytic subunit (CDK) that is present throughout the cell cycle and an activating subunit (cyclin) that is present only at specific stages of the cell cycle.[2] The importance of CDKs in cell cycle regulation and the frequent documented deregulation of CDKs and their modulators in cancer have stimulated great research efforts in this area.[3] In particular, the D type cyclins, D1, D2, and D3, associated with CDK 4 and 6 are believed to play a critical role early in the G1 phase of the cell cycle. These complexes phosphorylate the retinoblastoma protein and inactivate its ability to act as a transcriptional repressor in a complex with E2F.[4a] Thus, inhibitors of these cyclin-dependent kinases that stop uncontrolled tumor cell growth are expected to be promising new therapeutic agents for the treatment of cancer.

During the past decade, several scaffolds were discovered with inhibitory activity against CDK.[4] Of these small-molecule CDK inhibitors, three compounds (flavopiridol,[5] UCN-01,[6a] and roscovitine[6b]) have entered into clinical development as cancer therapeutics. Despite the disclosure of many potent CDK inhibitors in the literature, most of them are potent inhibitors against CDK1 and/or CDK2.[7−17] Owing to recent strong evidence of the link between D1/CDK4 activity and many types of tumors, there is significant interest in searching for selective CDK4 inhibitors. However, a few selective CDK4 inhibitors have recently been reported in the literature.[18−25]

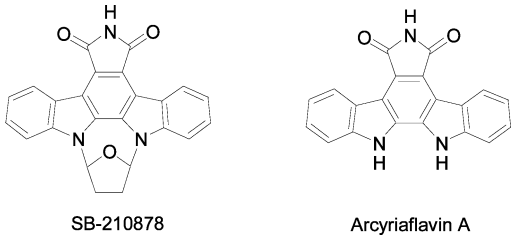
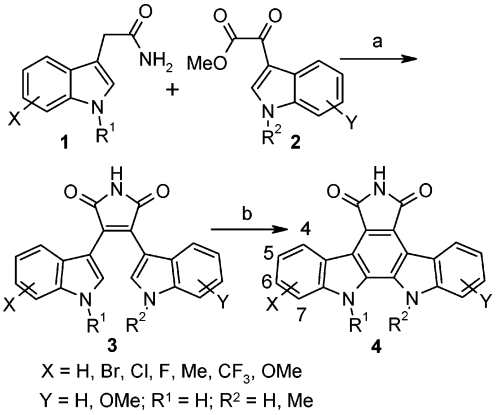


Figure 1.

Scheme 1. Synthesis of the Unsymmetrical Indolocarbazoles



[a] (a) KO^{t}Bu, THF, room temperature; (b) DDQ, solvent, *p*-TsOH, reflux, or I_2, benzene, *hν*.

High-throughput screening of the Lilly compound collection against the cyclin D1/CDK4 enzyme complex yielded several interesting scaffolds, including SB-210878 (Figure 1), with an IC_{50} of 0.54 μM in the D1/CDK4 kinase assay. Structurally, SB-210878 belongs to the indolocarbazole family, and subsequent testing of other indolocarbazoles indicated that the naturally occurring arcyriaflavin A (Figure 1) also showed moderate inhibitory activity against D1/CDK4. Because of our interest in obtaining selective and potent CDK4 inhibitors, we began a medicinal chemistry approach around this lead with the help of a molecular modeling study using a CDK4 homology model. In this report, we discuss the synthesis and structure−activity relationships of unsymmetrical substituted indolocarbazoles as D1/CDK4 inhibitors.

Chemistry. The general synthetic approach to the unsymmetrically substituted indolocarbazole compounds **4** is outlined in Scheme 1. The key step in the synthesis of these compounds involved the cyclization of bisindolylmaleimide **3** to the corresponding indolocarbazole **4**. This was accomplished by DDQ-mediated oxidative cyclization in the presence of a catalytic amount of *p*-toluenesulfonic acid using benzene, toluene, or dioxane as solvent.[26] Alternatively, the cyclization of **3** could be carried out under photochemical conditions using

* To whom correspondence should be addressed. Phone: 317-433-3697. Fax: 317-277-2035. E-mail: Zhu_Guoxin@lilly.com.

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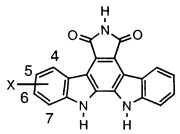
Table 1. Effect of Bromine Substitution on D1/CDK4 Activity[29] and Kinase Selectivity

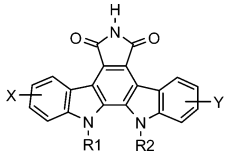


		IC$_{50}$ (μM)				
		D1-CDK4 RB21	E-CDK2 RB21	B-CDK1 histone	CamKII autocamtide	PKA histone
4a	H	0.16	0.53	1.1	<0.125	>2.0
4b	4-Br	0.08	0.22	0.3	0.058	0.103
4c	5-Br	4.4	4.3	19.4	<1.25	>20.0
4d	6-Br	0.076	0.52	2.1	12.4	>20.0
4e	7-Br	16.2	19.4	>20.0	11.1	>20.0

iodine as an oxidant in a benzene/methanol solvent combination.[27] **3** was prepared in good yield via base-promoted condensation of an appropriately substituted indolyl 3-acetamide **1** and an indolyl 3-glyoxylate **2** in the presence of KO^{t}Bu in THF based on Faul's procedure.[28] In some cases, dehydration of the hydroximide intermediates was slow under basic conditions, so concentrated hydrochloric acid was added to accelerate the dehydration of this intermediate. Both **1** and **2** are readily available by a simple transformation from a variety of commercially available indoles following literature procedures.[28]

Results and Discussion. Selective Inhibition of D1/CDK4 by Indolocarbazole Analogues. The initial medicinal chemistry effort was focused on evaluating the effect of substitution at the different positions of the indole in the indolocarbazole on the D1/CDK4 inhibitory activity as well as selectivity against the other kinases. The D1/CDK4 enzyme inhibitory activities of indolocarbazoles **4** was determined by measuring the phosphorylation of Rb protein.[29] These compounds were also tested in other kinase inhibitory assays by measuring the phosphorylation of the corresponding substrates (e.g., E-CDK2 using Rb21 as substrate, B-CDK1 and PKA using histone as substrates, CamKII using autocamtide as substrate, and GSK3β using pCREB as substrate). Staurosporine, the well-known kinase inhibitor, was used as a standard compound for the assays. Table 1 summarizes the enzyme inhibitory activity of indolocarbazoles substituted by bromine at the different indole positions. **4c** and **4e** with bromine at the 5- and 7-position, respectively, of one indole led to poor inhibitory activity against D1/CDK4. While bromine at the 4- and 6-position of the indole in the indolocarbazole are well tolerated for D1/CDK4 inhibitory activity, both **4b** and **4d** showed a 2-fold activity increase against D1/CDK4 compared to parent indolocarbazole, arcyriaflavin A. Besides the potent activity against D1/CDK4, compound **4b** with a bromine at the 4-position of an indole was also very potent against other kinases (e.g., IC$_{50}$ of 220 nM for E-CDK2, 300 nM for B-CDK1, 58 nM for CamKII, and 103 nM for PKA). However, **4d** with a bromine at the 6-position of an indole was significantly more selective against other kinases tested; **4d** was 7-fold less active against cyclin E/CDK2. Similar trends were also found when fluorine or chlorine was used instead of bromine as the substituted group on one of the indoles. **4a**−**e** were found to be inactive against GSK3β at 20 μM. Thus, substitution on the 5- or 7-positions of the indolocarbazole decreases D1/CDK4 inhibitory activity and the indolocarbazole analogues with a substitution at the 4- or 6-position improve D1/CDK4 activity. However, 6-substituted indolocarbazoles gave better kinase selectivity than the corresponding 4-substituted analogues.

Table 2. Kinase Inhibitory Activity of 6-Substituted Indolocarbazoles



					IC$_{50}$ (μM)			
compd **4**	X	R1	R2	Y	D1-CDK4 RB21	E-CDK2 RB21	CamKII autocamtide	PKA histone
4f	Br	H	Me	H	0.074	nt	0.174	>2.0
4g	F	H	H	H	0.103	0.141	0.092	>2.0
4h	F	H	Me	H	0.042	0.144	>2.0	>2.0
4i	Cl	H	H	H	0.072	0.278	0.914	>2.0
4j	Me	H	H	H	0.226	>1.0[a]	0.082	>2.0
4k	CF_3	H	H	H	1.135	>2.0[a]	>2.0	>2.0
4l	OMe	H	H	H	0.134	2.287[a]	0.236	>2.0
4m	Br	H	H	OMe	0.63	>2.0[a]	>2.0	>2.0

[a] Rb^{ING} as substrate.

Table 3. Cellular Activity for Substituted Indolocarbazoles

	compd										
	4a	**4b**	**4d**	**4f**	**4g**	**4h**	**4i**	**4j**	**4k**	**4l**	**4m**
HCT-116 IC$_{50}$ (μM)	0.85	0.26	2.23	2.36	3.53	0.76	2.47	4.54	1.65	1.79	2.64
NCI-H460 IC$_{50}$ (μM)	0.59	0.71	2.76	1.23	2.60	0.53	1.62	4.22	1.35	1.18	1.78

After finding out that the 6-bromo-substituted indolocarbazole **4d** had the most potent D1/CDK4 enzyme inhibitory activity with the desired selectivity profile within the kinase panel tested, we conducted an SAR study on the 6-position of the indole by introducing different functional groups at this position. The enzymatic kinase activities of these 6-substituted indolocarbazoles are summarized in Table 2. As shown in Table 2, a variety of substituents at the 6-position of the indole was well tolerated for maintaining D1/CDK4 activity; all of them exhibited potent D1/CDK4 inhibitory activity. In general, these compounds were more potent against D1/CDK4 compared to E/CDK2 and PKA. However, the selectivity against CamKII was more sensitive to the nature of the substituent group. Substituting the 6-position of one indole in the carbazoles with fluorine (**4g**), methyl (**4j**), or methoxy (**4l**) led to potent CamKII activity. By introducing a methyl at one of the indole nitrogen R2 positions, we were able to improve the potency of D1/CDK4 and selectivity against CamKII (**4g** vs **4h** in Table 2). A combination of 6-bromo and 6′-methoxy substitution (**4m**) was also evaluated. Although D1/CDK4 activity was diminished, better selectivity against other panel kinases was observed. **4f**−**m** were found to be much less active against GSK3β compared with their inhibitory activity against D1/CDK4. They were found to be inactive against GSK3β at 5 μM.

Kinetic studies showed that **4d**,**g**,**i**,**l** exhibited pure competitive inhibition with respect to ATP; thus, certain 6-substituted indolocarbazoles were potent and selective ATP competitive inhibitors of D1/CDK4.

Inhibition of Tumor Cell Proliferation and Effect on Cell Cycle and pRb Phosphorylation. In addition to the kinase activity discussed above, the synthesized indolocarbazoles were also studied for their

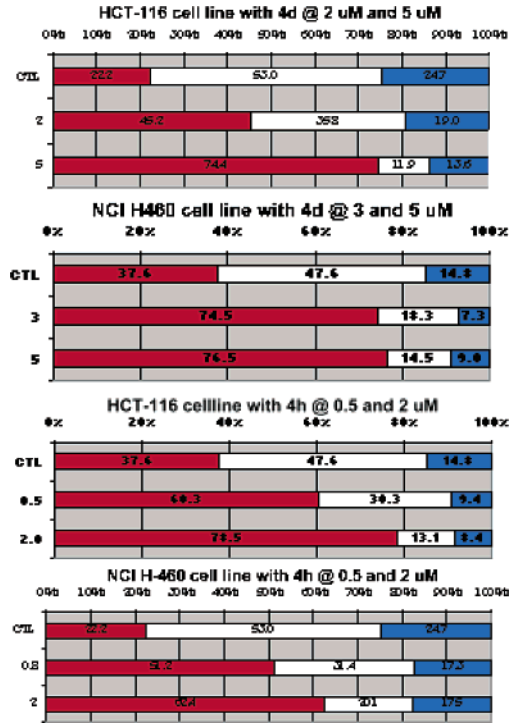


Figure 2. Cell cycle analysis of the cell lines treated for 24 h with D1/CDK4 inhibitors **4d** and **4h**: (red) G1; (white) S; (blue) G2/M.

antiproliferative activity in a variety of tumor cell lines.[30] The properties of these indolocarbazole analogues in in vitro cellular activities in human colon carcinoma (HCT-116) and non-small-cell lung carcinoma (NCI-460) are shown in Table 3. Indolocarbazole analogues **4** inhibited cell growth in the HCT-116 and NCI-H460 tumor cell lines with IC_{50} values from 0.26 to 4.54 μM.

It is clear that CDK4 plays a critical role in the G1-S transition of the cell cycle by phosphorylating Rb. It is also known that the Ser-780 residue on Rb is specifically phosphorylated by CDK4.[31] Thus, inhibition of cellular CDK4 activity will result in the inhibition of Rb phosphorylation on Ser-780 and cell cycle arrest in the G1 phase. To investigate whether these CDK4 inhibitors will produce G1 cell cycle arrest and inhibition of Rb phosphorylation, we evaluated the effects of these compounds by cell cycle analysis and a phosphorylation assay in tumor cells.

A cell cycle inhibition study was conducted by treating HCT-116 and NCI-460 cells with different concentrations of the CDK inhibitors for 24 h and then analyzing them by flow cytometry. As exemplified in Figure 2, treatment of the tumor cells with these indolocarbazole D1/CDK4 inhibitors results in a significant accumulation of cells in the G1 population and decreases the S and G2/M populations in a dose-responsive manner. In most cases, G1 arrest occurred in the same concentration range as that for the corresponding cell growth inhibition (cellular inhibition IC_{50}).

To this point, we have discussed that the 6-substituted indolocarbazoles are potent D1/CDK4 selective inhibitors in vitro and arrest human tumor cells in the G1 phase. We also evaluated the effects of the compounds on CDK4 activity by measuring phospho-Rb^{S780} levels using a phosphospecific antibody. Colon carcinoma cells (HCT-116) cells were treated with compounds at 1× and 3× antiproliferation IC_{50} concentrations for 24 h, followed by the Western blot analysis. As shown in Table 4, these compounds potently inhibited phosphorylation of Rb^{S780}, demonstrating that they inhibit cellular CDK4 activity. These specific observations of the G1 cell cycle arrest and inhibition of phosphorylation of serine 780 on pRb indicate that these indolocarbazoles are potent CDK4 inhibitors.

Table 4. pRb Phosphorylation Inhibition by Indolocabazoles[a]

	compd							
	4b	**4f**	**4g**	**4h**	**4i**	**4k**	**4l**	**4m**
HCT-116 IC_{50} (μM)	0.26	2.36	3.53	0.76	2.47	1.65	1.79	2.64
% pRb inhibition @ IC_{50}	33	86	66	82	72	82	66	85
% pRb inhibition @ 2 IC_{50}	56	85	89	86	92	97	82	93

[a] Colon carcinoma cells (HCT-116) treated with compounds at 1× and 3× antiproliferation IC_{50} concentration for 24 h.

Finally, selected compounds were studied in the human colon carcinoma (HCT-116) xenograft to evaluate their antitumor effects. These compounds caused a certain degree of tumor growth delay in this model (data not included). These results indicated that these small molecular D1/CDK4 inhibitors might have therapeutic potential in cancer treatment.

In summary, we have disclosed 6-substituted indolocarbazoles representing a new class of D1/CDK4 inhibitors. These compounds are selective and ATP competitive D1/CDK4 inhibitors and are capable of inhibiting cell growth in the human tumor cell lines HCT-116 and NCI H460. In addition, the specific G1 cell cycle arrest and selective inhibition of phosphorylation of serine 780 on pRb are consistent with the in vitro D1/CDK4 inhibitory activity.

Supporting Information Available: Experimental details for the assays and the final compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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