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Aryl[a]pyrrolo[3,4-c]carbazoles as Selective Cyclin D1-CDK4 Inhibitors

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Abstract—The synthesis of new analogues of Arcyriaflavin A in which one indole ring is replaced by an aryl or heteroaryl ring is described. These new series of aryl[a]pyrrolo[3,4-c]carbazoles were evaluated as inhibitors of Cyclin D1-CDK4. A potent and selective D1-CDK4 inhibitor, **7a** (D1-CDK4 IC₅₀ = 45 nM), has been identified. The potency, selectivity profile against other kinases, and structure–activity relationship (SAR) trends of this class of compounds are discussed.

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Cyclin–CDK complexes regulate the progression of cells through the cell cycle.¹ To date, strong evidence suggests a G1-phase role for D-type cyclins through association with CDK4 and CDK6. CDK2 complexes with cyclin E and participates in the G1/S transition, whereas association with cyclin A peaks at S phase and may have a profound effect on proliferation. Moreover, CDK1 (Cdc2) plays an essential role in mitosis by interaction with B-type cyclin in the G2/M transition. Aberrations in CDKs and their regulators have been found in a large percentage of human tumors, including melanoma, lymphoma and carcinomas of the breast, lung, ovary, oropharyngeal and colon.² Thus, inhibitors of these cyclin–CDK complexes might have a broad range of therapeutic applications in cancer as well as other diseases.

In the past decade, many small molecules have been reported as CDK inhibitors. Among these, the microbial alkaloids, staurosporine **1** and UCN-01 **2**, have

been identified as two of the most potent CDK inhibitors.³ However, they lack selectivity against other kinases such as PKC or CAMKII. On the other hand, flavopiridol,⁴ butyrolactone,⁵ paullones,⁶ indirubins,⁷ oxindoles,⁸ hymenialdisine,⁹ 2,6,9-tri-substituted purines,¹⁰ indenopyrazoles,¹¹ β -carboline,¹² and 2-benziliden-benzofuranones¹³ have been shown to be relatively specific for certain CDKs (CDK1 and CDK2) without having significant CDK4 inhibitory activity except for flavopiridol. The synthesis of selective and ATP competitive inhibitors of cyclin D1-CDK4 is less prevalent. To the best of our knowledge, fascaplysin, a marine natural product,¹⁴ 5-arylamino-4,7-dioxobenzothiazoles,¹⁵ benzocarbazoles,¹⁶ pyrido[2,3-*d*]pyrimidin-7-ones,¹⁷ cinnamaldehydes¹⁸ and diarylureas,¹⁹ have been described as selective CDK4 inhibitors (IC₅₀ ranging from 0.002 to 7.5 μ M). However, only in vitro activities have been disclosed for these inhibitors (Fig. 1).

In the previous paper, we reported the structure-based inhibitory activity of indolyl[2,3-*a*]pyrrolo[3,4-*c*]carbazoles versus bis-indolylmaleimides against cyclin D1-CDK4.²⁰ Based upon these data, our main objective was to obtain new cyclin D1-CDK4 inhibitors that were

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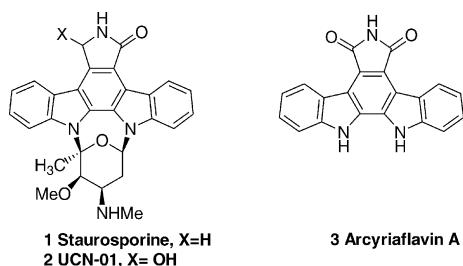


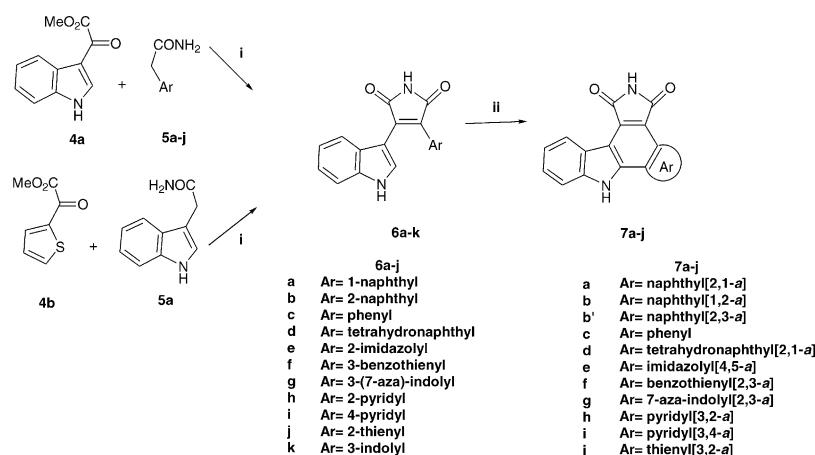
Figure 1. Chemical structures for staurosporine, UCN-01 and arcyriaflavin A.

more selective than staurosporine. The preserved maleimide moiety possesses the ability to function as the hydrogen bond donor–acceptor pair in the ATP-binding pocket of the D1–CDK4 enzyme complex. Since the maleimide region seems to be critical for potency, we envisioned that replacement of an indole moiety of indolocarbazole **3** with a variety of aryl or heteroaryl rings might lead to new, structurally diverse carbazole analogues of staurosporine with a selective profile against CDK4.

The preparation of these aryl[*a*]pyrrolo[3,4-*c*]carbazoles was accomplished using a general synthetic route, that relied on the ring closure of 3-aryl-4-indolyl maleimides. Maleimides **6a–k** were prepared in good yields by condensation of indolyl-glyoxylate **4a** with the complementary aryl-acetamide **5a–j** (Scheme 1), following a procedure reported by Faul et al.²¹ However, maleimide **6j** was prepared by condensation of the thienyl-glyoxylate **4b** and the indole-acetamide **5a**. In two specific cases, **6g** and **6j**, elimination of the hydroxy-succinimide intermediate did not take place under the standard conditions. The elimination of the hydroxy functionality was accomplished using hydrochloric acid in DMSO to obtain maleimide **6g**, and using DMAP-carbonyl diimidazole in acetonitrile, for maleimide **6j**. Cyclization of these maleimides to the corresponding aryl[*a*]pyrrolo[3,4-*c*]carbazoles, **7a–j**, was the key step (Scheme 1). Indolocarbazole **3** was obtained from maleimide **6k** using *p*-toluene sulfonic acid as the catalyst for cyclization.²² Unlike indolocarbazole **3**, the other carbazoles were obtained by photochemical oxidative ring closure pro-

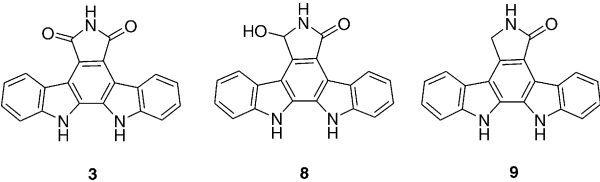
cedures using either benzene, dioxane or acetone as solvent, and iodine, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) or acetone as the oxidative agent (Scheme 1).²³ It is worth noting that the cyclization of maleimide **6b** gave two different carbazoles depending on the cyclization conditions. While photochemical conditions gave the down-angular carbazole **7b**, the use of palladium acetate in refluxing acetic acid produced, albeit in low yield, the linear carbazole **7b'** (Scheme 1). Finally, carbazoles **8** and **9** were prepared from compound **3** by partial reduction of the maleimide moiety following literature procedures.²⁴

Aryl[*a*]pyrrolo[3,4-*c*]carbazoles **3**, **7a–j**, **8** and **9** were evaluated for their D1–CDK4 inhibitory activity in an enzymatic assay, by measuring phosphorylation of the Rb^{ING} substrate according to standard protocols.²⁵ In addition, other assays such as B–CDK1, E–CDK2, PKA, PKC and CAMKII were also conducted to determine the selectivity profile.²⁶ The IC₅₀ values determined are compared with those of staurosporine used as control, and are summarized in Tables 1–3. As reported in the literature, staurosporine was found to be a potent and nonselective kinase inhibitor. Although staurosporine potently inhibits D1–CDK4 (IC₅₀ = 59 nM), it also potently inhibits other kinases tested (E–CDK2, B–CDK1, CAMKII, PKA and PKC, Table 1). However, it was found that the simple and symmetric indolocarbazole **3** showed good inhibitory activity (IC₅₀ = 140 nM) for D1–CDK4 and most interestingly, it demonstrated significantly improved selectivity towards several other kinases (B–CDK1, E–CDK2, PKA and PKC) (Table 1). To investigate the importance of the maleimide functionality in compound **3**, reduction of one carbonyl group either partially to the secondary alcohol (compound **8**), or fully to the methylene unit (compound **9**) was performed. As the data in Table 1 indicate, maleimide modification was not well tolerated. Indolocarbazoles **8** and **9** showed 27- and 6-fold less potency, respectively, compared to indolocarbazole **3**. This result suggests that in the absence of the deoxy-sugar moiety, both carbonyl groups in the maleimide ring of the indolocarbazoles are involved in the binding and inhibitory activities towards the D1–CDK4 enzyme.



Scheme 1. (i) KOtBu, THF, 0 °C to rt; (ii) C₆H₆, I₂, *hν*, reflux or Dioxane, DDQ, *hν*, reflux or acetone, *hν*, 35 °C or Pd(Ac)₂, HOAc.

Table 1. Enzyme inhibition of indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazoles (IC₅₀ μM)

						
Compd	D1-CDK4 (Rb ^{ING})	B-CDK1 (Histone)	E-CDK2 (Rb ^{ING})	CAMKII (Histone)	PKA (Histone)	PKC
1	0.059	0.012	0.012	0.002	0.016	0.007
3	0.14	1.13	0.90	0.025	> 2.0	> 100
8	3.83	NT	NT	NT	NT	NT
9	0.83	NT	> 1.0	0.184	> 2.0	NT

NT, not tested.

Table 2. Enzyme inhibition of aryl[*a*]pyrrolo[3,4-*c*]carbazoles (IC₅₀ μM) **7a–l** against cyclin D1-CDK4

Compd	Ar	D1-CDK4 (Rb ^{ING})
7a	Naphthyl[2,1- <i>a</i>]	0.045
7b	Naphthyl[1,2- <i>a</i>]	0.43
7b'	Naphthyl[2,3- <i>a</i>]	> 10
7c	Phenyl	0.26
7d	Tetrahydronaphthalenyl[2,1- <i>a</i>]	0.41
7e	Imidazolyl[4,5- <i>a</i>]	0.74
7f	Benzothienyl[2,3- <i>a</i>]	0.22
7g	7-Aza-indolyl[2,3- <i>a</i>]	0.25
7h	Pyridyl[3,2- <i>a</i>]	0.18
7i	Pyridyl[3,4- <i>a</i>]	1.81
7j	Thienyl[3,2- <i>a</i>]	0.15

To further investigate how the molecular symmetry of the carbazole region could modify the D1-CDK4 inhibitory activity, one of the indole rings of **3** was replaced by a different aryl or heteroaryl moiety. It was found that almost all exhibited good activity towards D1-CDK4 with inhibitory potency similar to that of arcylriaflavin A (**3**) (Table 2).

Activity of the naphthylcarbazoles **7a–b'** (Fig. 2) turned out to be quite intriguing. The orientation of the naphthyl group and how it fused to the indolocarbazole moiety greatly affected the D1-CDK4 inhibitory activity of these compounds. The up-angular derivative **7a** was found to be as active as staurosporine (IC₅₀ 45 nM) and clearly selective against other kinases (Table 3). Kinetic analysis showed that **7a** exhibited pure competitive inhibition with respect to ATP, and is not competitive with the peptide substrate (data not included). However, the other two regioisomers, **7b** (down-angular) and

Table 3. Kinase selectivity profile for naphthylcarbazole **7a**

Kinase	IC ₅₀ , μM
D1-CDK4 (Rb ^{ING})	0.045
E-CDK2 (Rb ^{ING})	0.275
B-CDK1 (histone)	0.590
PKA (histone)	> 20
PKCα	52.75
PKCβII	9.0
PKCγ	56.5
GSK3β (pCreb)	4.38

7b' (linear) exhibited a different activity profile than compound **7a**. For example, compound **7b** is 10-fold less effective as an inhibitor towards D1-CDK4 (IC₅₀ 430 nM) than **7a**, whereas compound **7b'** was basically inactive (IC₅₀ > 10 μM). This result suggested that these naphthylcarbazoles could bind differently in the ATP pocket. The different orientations of the naphthyl ring in relation to the maleimide warhead preclude the effective interaction of certain regioisomers with its kinase target. Replacement of the naphthyl moiety by a phenyl ring, **7c**, or partial reduction of the naphthyl ring to the tetrahydro analogue **7d**, resulted in a loss of potency relative to **7a** (Table 2). Presumably, the lack of potency of carbazoles **7b–d** is due to the loss of the naphthyl-protein backbone interaction.

We have also studied the replacement of the indole moiety by other heterocyclic rings, **7e–j**. It was found that replacing the indole with different small heteroaromatic rings such as imidazolyl **7e**, pyridyl **7h–i**, thienyl **7j**, or bicyclic heterocycles such as benzothienyl, **7f** or 7-aza-indole, **7g**, led to a decrease in D1-CDK4 inhibitory activity (Table 2) compared to naphthylcarbazole **7a**.

In addition to the kinase inhibition studies, effects on the cell proliferation *in vitro* were determined in two human carcinoma cell lines, HCT-116 (colon) and NCI-H460 (lung) (Table 4).²⁷ These cell lines were selected based on their expression of Rb protein, cyclin D1 and CDK4 determined by Western blotting.²⁸ The data reported in Table 4 indicates that antiproliferative activity was achieved with these aryl carbazoles with IC₅₀'s ranging from 0.59 to 5.83 μM.

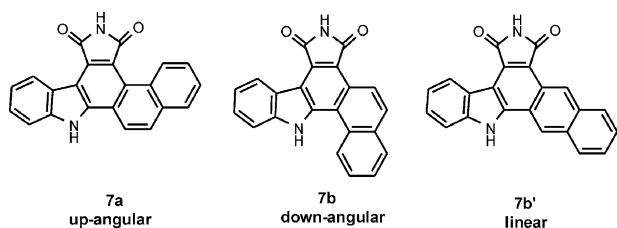
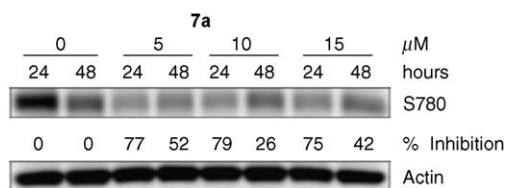
**Figure 2.** Chemical structures of naphthyl[*a*]pyrrolo[3,4-*c*]carbazoles.

Table 4. Effect on cell proliferation of aryl[a]pyrrolo[3,4-c]carbazoles (IC₅₀, μ M)

Compd	HCT-116	NCI-H460
3	0.85	0.59
7a	5.05	2.11
7b	> 10	> 10
7c	5.83	3.42
7f	3.02	2.84
7g	1.05	1.16
7h	> 10	> 10
7j	> 10	> 10
9	1.72	3.20

**Figure 3.** Inhibition of Rb (Ser780) phosphorylation in HCT-116 cells by **7a**.

Analysis of selected arylcarbazoles **3**, **7a** and **9** by flow cytometry (FCM) in NCI-H460 (lung, p16⁻, Rb⁺) and HCT-116 (colon, p16⁻, Rb⁺) cell lines were done according to standard protocols.²⁹ This analysis showed different behaviors depending on the nature of maleimide moiety. The FCM showed that indolocarbazole **3** caused an increase in the G1 population in a dose-dependent manner (from 27 to 64%) when HCT-116 cells were treated at 4.5 μ M (5 fold IC₅₀). In contrast, indolocarbazole **9** caused a maximum G1 accumulation of 35.0% at 1.7 μ M concentration while high doses (5 μ M) produced the opposite effect (91.8% in G2/M arrest). On the other hand, despite its low anti-proliferative activity, compound **7a** caused a clear maximum G1 accumulation of 85.3% versus control (36.6%) at a 5 μ M concentration in the NCI-H460 cell line. No correlation is observed between the cyclin D1/CDK4 potency and the anti-proliferative potential; however, G1 arrest is observed only with the more potent CDK4 inhibitors. The lack of correlation and an apparent threshold for effect may result from the poor solubility of these planar structures.

Finally, we evaluated the effects of compound **7a** on CDK4 activity by measuring phospho-RbS⁷⁸⁰ levels using a phospho-specific antibody.³⁰ Colon carcinoma cells (HCT-116) were treated with compound **7a** at the anti-proliferation IC₅₀ concentration for 24 and 48 h, followed by Western Blot analysis (Fig. 3). This study showed that compound **7a** potently inhibited phosphorylation of Rb₇₈₀, consistent with its cyclin D1–CDK4 inhibitory activity.

Our sar studies have shown that both the maleimide ring and the aryl fused at the [a]-position are critical requirements for D1–CDK4 inhibitory activity. We have identified a simpler analogue, **7a**, which shows potent and selective D1–CDK4 inhibitory activity. Although **7a** showed favorable in vitro potency and

selectivity versus D1–CDK4, additional SAR studies directed towards increasing activity in cell-based assays would be required before further advance to in vitro and in vivo studies.

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