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Studies on Cyclin-Dependent Kinase Inhibitors: Indolo-[2,3-*a*]pyrrolo[3,4-*c*]carbazoles versus Bis-indolylmaleimides

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Abstract—A series of indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazoles and their bis-indolylmaleimides precursors have been prepared in order to compare their activity as D1–CDK4 inhibitors. Both enzymatic and antiproliferative assays have shown that the structurally more constrained indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazoles are consistently more active (8–42-fold) in head-to-head comparison with their bis-indolylmaleimides counterparts. Cell-cycle analysis using flow cytometry have also shown that the indolocarbazoles are selective G1 blockers while the bis-indolylmaleimides arrest cells in the G2/M phase.

Cyclin-dependent kinases (CDKs) have recently raised considerable attention because of their central role in the regulation of cell-cycle progression.[1] A high incidence of genetic mutation of CDK substrates and deregulation of CDK modulators were found in a number of disease states, particularly in cancer.[2] One of the best defined mechanisms triggering entry into the cell cycle is the production of cyclin D1 in response to growth factors in quiescent cells or due to alterations in the cell cycle machinery in cancer.[3] Cyclin D1–CDK4 phosphorylates Rb blocking its repression of the E2F/DP dimer.[4] This allows E2F/DP dependent transcription of cyclin E and other genes associated with entry into S phase.[5] Taking into consideration the essential role of CDKs in the cell cycle and the strong link between CDKs and many human tumors, a substantial research effort has been directed to search for selective CDK inhibitors as anticancer agents.[6] We describe compounds which inhibit cyclinD1–CDK4, arrest cells in G1-phase, and block the phosphorylation of Rb.

A variety of CDK pharmacophores have been described in the past decade, most of them as potent inhibitors against CDK1 and/or CDK2.[6d] In this context, the microbial indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole alkaloids are one of the most relevant CDK pharmacophores studied. Staurosporine **1**, has been identified as one of the most potent (but non-selective) CDK inhibitors. The same activity for the natural PKC inhibitors **2**–**4a**[7–9] is rarely mentioned.[10] On the other hand, SB-218078[11] **5**, a ChK1 and PKC inhibitor, and structurally related to K-252a **2**, has been identified from our HTS as a D1–CDK4 inhibitor. Indolocarbazoles **2**–**4a** also showed moderate inhibitory activity against D1–CDK4 (Table 1) in our isolated enzyme assay (Fig. 1).

Indolocarbazoles **3**, **4a** and **5** showed 28-, 5- and 18-fold less potency, respectively, compared to indolocarbazole **2**. In all the cases, some selectivity versus E–CDK2 inhibition was observed, especially for compounds **2** and **4a**. A statistical analysis of our database shows that IC_{50} differences greater than 3.2-fold for CDK4, 2.3-fold for CDK2, 6.1-fold for HCT-116, and 4.1-fold for NCI-H460 are significant.[12] In addition, compounds **2**–**5** also showed antiproliferative activity in two human carcinoma cell lines (HCT-116 colon and NCI-H460 lung) in

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Table 1. Inhibitory activities of indolocarbazoles **2**–**5** towards D1–CDK4, E–CDK2, and in vitro antiproliferative activity (IC_{50} μM)

Compd	D1–CDK4 (Rb^{ING})	E–CDK2 (Rb^{ING})	HCT-116	H460
2	0.03	0.26	0.16	0.24
3	0.83	>1.0	1.72	3.20
4a	0.14	0.90	0.85	0.59
5	0.54	0.68	0.70	1.64

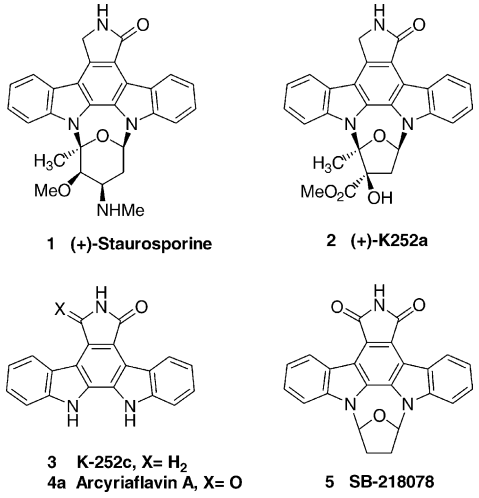
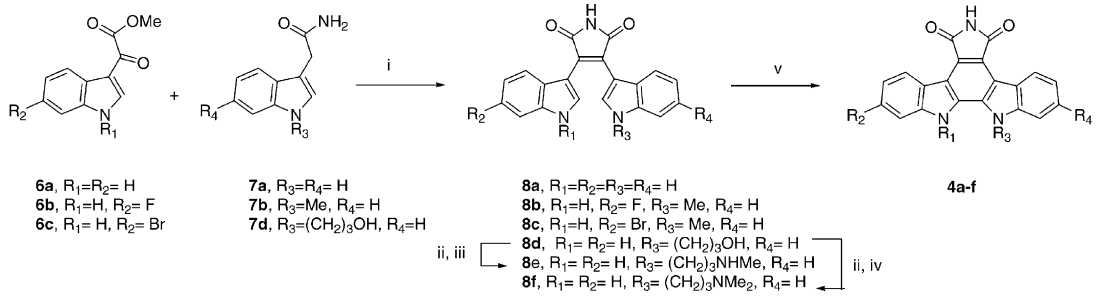


Figure 1. Chemical structures for staurosporine, K-252a, K-252c, arcyriaflavin A and SB-218078.

in vitro cellular assays (Table 1). A cell-cycle inhibition study conducted by treating HCT-116 and NCI-H460 cells with different concentrations of carbazoles **2**–**5** produced intriguing results. While indolocarbazoles **2** and **3** (non-symmetrical) produced a significant accumulation of cells in the G2 population, the symmetric **4a** and **5** caused a G1 arrest in a dose-responsive manner (data not shown). It is not clear whether inhibition of CDK4 and CDK2 or CDK4 alone is ideal for antiproliferation; however, recent work by Tetsu and McCormick[13] has suggested CDK2 may not be essential for cell cycle progression. Furthermore, a review by Sears and Nevins[14] suggests that CDK2 is not required for entry into S phase. Given the proposed redundancy in phosphorylation of Rb by both CDK2 and CDK4, either of which may relieve Rb supression of E2F/DP transcription inhibition of both CDK2 and CDK4 may provide a therapeutic benefit. These results suggest that CDK4 inhibition may provide therapeutic benefit in the treatment of cancer while the importance of the inhibition of CDK2 requires further investigation.

Based on our observations, we envisioned that (i) the substitution of the indole-nitrogen could modulate the inhibitory potency of the indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazoles, and (ii) the symmetry of the maleimide portion might play a key role in the inhibitory potency and cell cycle selectivity. At this point, we were interested in the comparison between the indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazoles and their bis-indolylmaleimide precursors. It is well known that bis-indolylmaleimides, and in general aryl-indolylmaleimides, strongly inhibit PKC but normally show little activity against many other kinases inhibited by staurosporine.[15] To extend the knowledge in this field, we report here the synthesis and a comparative study of a series of bis-indolylmaleimides and their corresponding indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazoles as D1–CDK4 inhibitors. In all the cases the maleimide heterocycle was conserved.

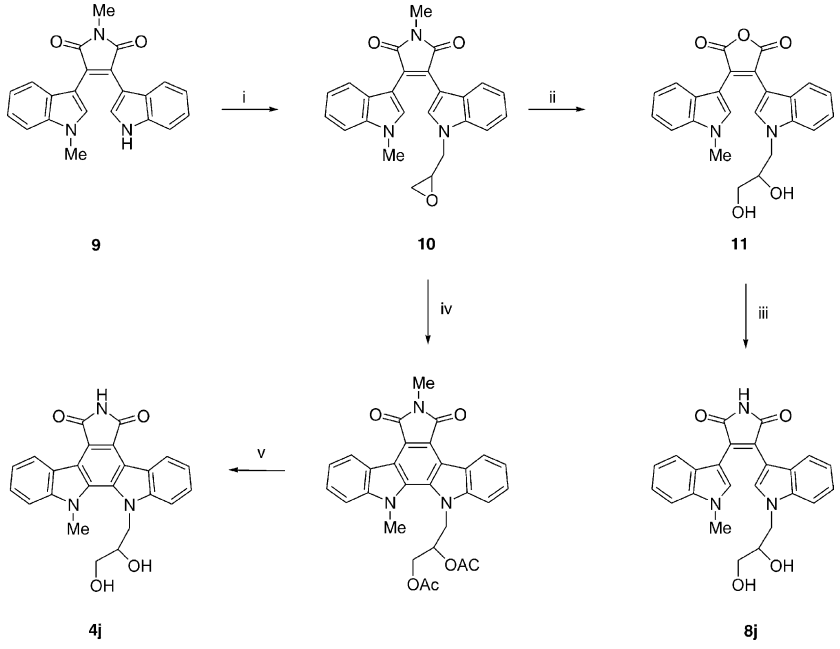
Indolocarbazoles **4** and their bis-indolyl maleimide precursors **8** were prepared as shown in Schemes 1 and 2. The methyl indolyl-glyoxylates **6** reacted with the complementary indolyl-acetamides **7** (Scheme 1) providing the corresponding bis-indolylmaleimides **8a**–**d**, following a procedure reported by Faul et al.[16] The amino derivatives **8e** and **8f** were prepared starting from the hydroxypropyl compound **8d**. Conversion to the corresponding mesyl derivative and displacement using methylamine or dimethylamine afforded **8e** and **8f**, respectively. Cyclization to the indolocarbazoles was achieved using *p*-toluenesulfonic acid (PTSA) and 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) in benzene under reflux for compounds **4a**–**c**,[17] palladium acetate in acetic acid for the hydroxypropyl compound **4d**, and using iodine in benzene under photochemical irradiation for the amino derivatives **4e** and **4f**. The bis-indolyl maleimides **8g**–**i** and their carbazoles **4g**–**i** were prepared following the literature.[18–20] Finally, indolocarbazole **4j** was prepared starting from the 6-*N*-methylmaleimide **9** (Scheme 2).[21] Conversion of **9** to the maleic anhydride after *N*-indole alkylation afforded intermediate **11**. Compound **11** was transformed to the corresponding maleimide **8j** under basic conditions. On the other hand, maleimide **10** was cyclized and deprotected affording the carbazole **4j**.



Scheme 1. (i) KO^{t}Bu, THF, 0 °C to rt; (ii) Ms_2O, pyridine; (iii) NH_2Me, dioxane; $(BOC)_2O$, Et_3N, DMF, rt; (iv) $NHMe_2$, dioxane; (v) *p*-TsOH, DDQ, C_6H_6, reflux or $Pd(OAc)_2$, AcOH, reflux or C_6H_6, I_2, *hν*, reflux.

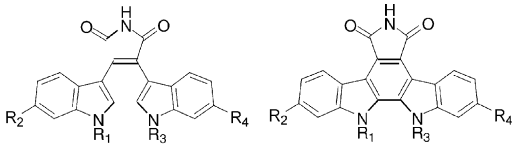
The inhibitory activity for compounds **4** and **8** against cyclin D1–CDK4 was evaluated in an enzymatic assay by measuring phosphorylation of Rb^{ING} substrate according to standard protocols (Table 2).[22] Inhibition of cyclin E–CDK2 and PKA were also measured to determine the selectivity profile.[17] In addition, effects on cell proliferation in vitro were evaluated in two human carcinoma cell lines, HCT-116 (colon) and NCI-460 (lung).[23]

Most of the compounds tested exhibited moderate inhibitory potency against cyclin D1–CDK4. Bis-indolylmaleimides **8a** and **8h** showed modest activity against D1–CDK4



Scheme 2. (i) Cs_2CO_3, DMF, $TsOH_2C$–  ; (ii) KOH (5N), EtOH; (iii) NH_4OH, DMF, 140 °C; (iv) $Pd(OAc)_2$, HOAc, reflux; (v) KOH (5 N), EtOH; HCl (5 N); NH_4OH, DMF, 140 °C.

Table 2. Kinase inhibitory activity (IC_{50}, μM) against D1–CDK4, E–CDK2 and PKA, antiproliferative activity (IC_{50}, μM) and cell-cycle effect of bis-indolylmaleimideas **8a–j** and indolocarbazoles **4a–j**



8a-j **4a-j**

Compd	R_1	R_2	R_3	R_4	CDK4 (Rb^{ING})	CDK2 (Rb^{ING})	PKA (histone)	HCT-116	H460	FCM HCT-116	FCM H460
8a	H	H	H	H	5.81	11.69	>2.0	—[a]	—	—	—
4a	H	H	H	H	0.14	0.90	>2.0	0.85	0.59	G1	G1
8b	H	F	Me	H	0.84	1.50	16.2	2.47	2.58	G2/M	G2/M
4b	H	F	Me	H	0.12	0.14	>2.0	0.76	0.53	G1	G1
8c	H	Br	Me	H	0.73	1.88	—	0.68	0.97	G2/M	G2/M
4c	H	Br	Me	H	0.11	>0.2	>2.0	2.36	1.23	—	G1
8d	H	H	$(CH_2)_3OH$	H	1.17	—	14.65	8.30	>10	—	—
4d	H	H	$(CH_2)_3OH$	H	0.07	0.18	>2.0	1.89	1.20	G1	G1
8e	H	H	$(CH_2)_3NHMe$	H	0.96	>1.0	3.52	>10	>10	—	—
4e[b]	H	H	$(CH_2)_3NHMe$	H	0.05	0.16	0.12	0.52	0.53	G1	G1
8f	H	H	$(CH_2)_3NMe_2$	H	0.99	10.18	>20	—	—	—	—
4f	H	H	$(CH_2)_3NMe_2$	H	0.13	>1.0	2.2	0.41	0.39	—	—
8g	Me	H	H	H	1.25	1.95	11.68	1.40	1.50	—	G2/M
4g	Me	H	H	H	0.08	>1	>20	0.51	0.43	G1	G1
8h	H	OMe	H	OMe	4.64	—	—	0.30	1.28	—	G2/M
4h	H	OMe	H	OMe	0.18	>1	>2.0	0.47	0.61	G1	G1
8i	Me	H	Me	H	0.89	0.58	>20	1.44	2.44	—	—
4i	Me	H	Me	H	>10	>10	—	>10	>10	—	—
8j	Me	H	$CH_2CHOHCH_2OH$	H	2.14	1.61	>20	6.60	6.60	—	—
4j	Me	H	$CH_2CHOHCH_2OH$	H	0.26	<0.06	0.81	0.82	0.71	G1–G2/M	G1

[a]Not tested.
[b]Hydroiodic salt.

($IC_{50} > 4$ μM), however the inhibitory activity increased when one or both indole-nitrogens supported an alkyl chain. The *N*-monomethyl derivatives **8b**, **8c**, **8g** increased the enzymatic inhibitory potency 5- to 8-fold compared with **8a**. The 3-hydroxypropyl, 3-methylaminopropyl and 3-dimethylaminopropyl mono-substituted analogues **8d–f**, also increased the inhibitory activity 5 to 6-fold as compared to **8a** in the D1–CDK4 assay. Alkylation of both indole-nitrogens was also investigated (Table 2). The introduction of two identical small groups, **8i**, produced a similar effect, increasing the potency 6-fold with respect to **8a**. However, substitution of one indole-nitrogen by a methyl group and the other by a polar group, **8j**, resulted in modest activity (2.14 μM). In general, these compounds were more potent against D1–CDK4 compared to E–CDK2 and PKA. Ring closure of these bis-indolylmaleimides **8** to their corresponding indolocarbazoles **4** led to a marked enhancement of the activity. The indolocarbazoles were systematically 6- to 42-fold more active in head to head comparison with their corresponding bis-indolylmaleimide precursors. For example, **4a** and **4h** were 42 and 26-fold more active than their precursors **8a** and **8 h,** respectively, against D1–CDK4. Following this similar pattern, the compared activity of the *N*-monomethylated analogues, **8b–c–g**, the 3-hydroxypropyl **8d**, or the 3-aminopropyl derivatives **8e–f**, showed that the corresponding indolylcarbazoles were 7- to 19-fold more active against D1–CDK4. A similar phenomenon was observed for the di-*N*-substituted derivatives **8j–4j**. The only exception to this fact was the *N*,*N*-dimethyl substituted pair **8i–4i** and was likely due to a very poor solubility of **4i**. Similar to the maleimides, all the indolocarbazoles except **4j** exhibited a greater potency against D1–CDK4 compared to E–CDK2. On the other hand, the selectivity against PKA was more sensitive to the nature of the alkyl group. Indolocarbazoles **4a–j** also exhibited selectivity versus B–CDK1.[24] This different behavior may be caused by the different flexibility of the indolocarbazoles versus the bis-indolylmaleimides. While a planar conformation with undistorted indole rings can be assumed for indolocarbazoles, NMR studies and X-ray analysis indicated that bis-indolylmaleimides could adopt different conformations due to the free rotation around the maleimide-indole single bond.

The properties of **4** and **8** in in vitro cellular activities in human colon carcinoma (HCT-116) and non-small-cell-lung carcinoma (NCI-H460) are shown in Table 2. Maleimides **8** inhibited cell growth in the HCT-116 and NCI-H460 tumor cell lines with IC_{50} values from 0.3 to 8.5 μM. However, indolocarbazoles **4**, in general demonstrated greater activity than their corresponding bis-indolylmaleimides with IC_{50} values from 0.4 to 2.4 μM.

Analysis of the indolocarbazole/bis-indolylmaleimide pairs **4**/**8** by flow cytometry (FCM) in NCI-H460 (lung, p16−, Rb+) and HCT-116 (colon, p16−, Rb+) cell lines were done according to standard protocols.[25] This analysis showed different behaviors depending on the constriction of the molecule. The FCM showed that bis-indolylmaleimides normally caused an increase of the G2-M population in a dose dependent manner when HCT-116 and NCI-H460 cells were treated with different concentrations of the CDK inhibitors for 24 h. Only compounds **8b**,**c** and **8g**,**h** could be used in this analysis due to the low antiproliferative activity that the bis-indolylmaleimides exhibited in these particular cell lines. Thus, maleimides **8g** and **8h** produced an increase in the G2/M population (from 15.1 to 69.9% and from 19.7 to 83.4%) at 3.9 μM (3- fold IC_{50}) and 7.5 μM (5 fold IC_{50}), respectively, in the NCI-H460 cell line. On the other hand, maleimides **8b** and **8c** caused a clear G2/M arrest (from 19.1 to 83.1% and from 17.5 to 95.3%, respectively) at 5.0 μM (2-fold IC_{50}) and 2.1 μM (3-fold IC_{50}), respectively, in the HCT-116 cell line. In contrast, all the indolocarbazoles caused a strong G1 arrest under the same conditions. For example, the unsubstituted indolylcarbazole **4a** caused a clear G1 arrest in a dose-dependent manner in both cell lines (28.1% to 63.5% at 4.5 μM (5-fold IC_{50}) for HCT-116 cell line and 41.4–64.2% at 3.0 μM (5-fold IC_{50}) for the H460 cell line). The monosubstituted indolylcarbazoles **4b–c** and **4g** also exhibited an increase in the G1 population with a corresponding decrease in the S and G2/M phases populations after 24 h in both cell lines. In the case of indolocarbazole **4i** it was not possible to make a determination, due to the high insolubility of this compound. The indolocarbazole **4j** showed a G1 effect at the IC_{50} concentration, but changed to a G2/M effect at high concentrations (3-fold IC_{50}) in the HCT-116 cell line. The G1 arrest observed for indolocarbazoles correlates with the potency shown against D1–CDK4 and E–CDK2.

Overall the IC_{50}'s for Cyclin D1–CDK4 correlated with the antiproliferative IC_{50} against HCT-116 ($R = 0.759$) and NCI-H460 ($R = 0.889$). Potent D1–CDK4 inhibitors (IC_{50} <200 nM) generally show a G1 arrest. The interpretation of these results are complicated by a correlation between inhibition of CDK2 and CDK4 activity ($R = 0.890$). In addition, treatment of HCT-116 cells with indolocarbazoles **4b–c**, **4e** and **4h** at 1×, 2× and 3× the antiproliferation IC_{50} concentration for 24 h, followed by Western blot analysis prevented hyperphosphorylation of Rb protein and also resulted in inhibition of, CDK4 specific, Ser780 Rb phosphorylation (Table 3). These observations suggest that the specific G1 cell cycle arrest caused by indolocarbazoles is consistent with the inhibition of CDK4 kinase.

The X-ray crystal structure of the human CDK2 and **4d** complex at 2.2 A resolution reveals the binding mode of these indolocarbazole inhibitors. Carbazole **4d** binds to the ATP binding site located in the cleft between the N- and C-terminal domains (Fig. 2a). The NH group from the carbazole forms hydrogen bond with the backbone carbonyl of Glu81. One of the carbonyl groups of the carbazole forms a hydrogen bond with the backbone amide NH of Leu83 (Val in CDK4); the other carbonyl group of the carbazole forms a hydrogen bond with a water molecule underneath Phe80. The hydroxyl group interacts with the side chain of Asn132. The other important residues in the binding pocket are: Phe82 (His in CDK4), Asp86, Gln131 (Glu in CDK4), and Glu145 (Fig. 2b). Structural and side chain differences

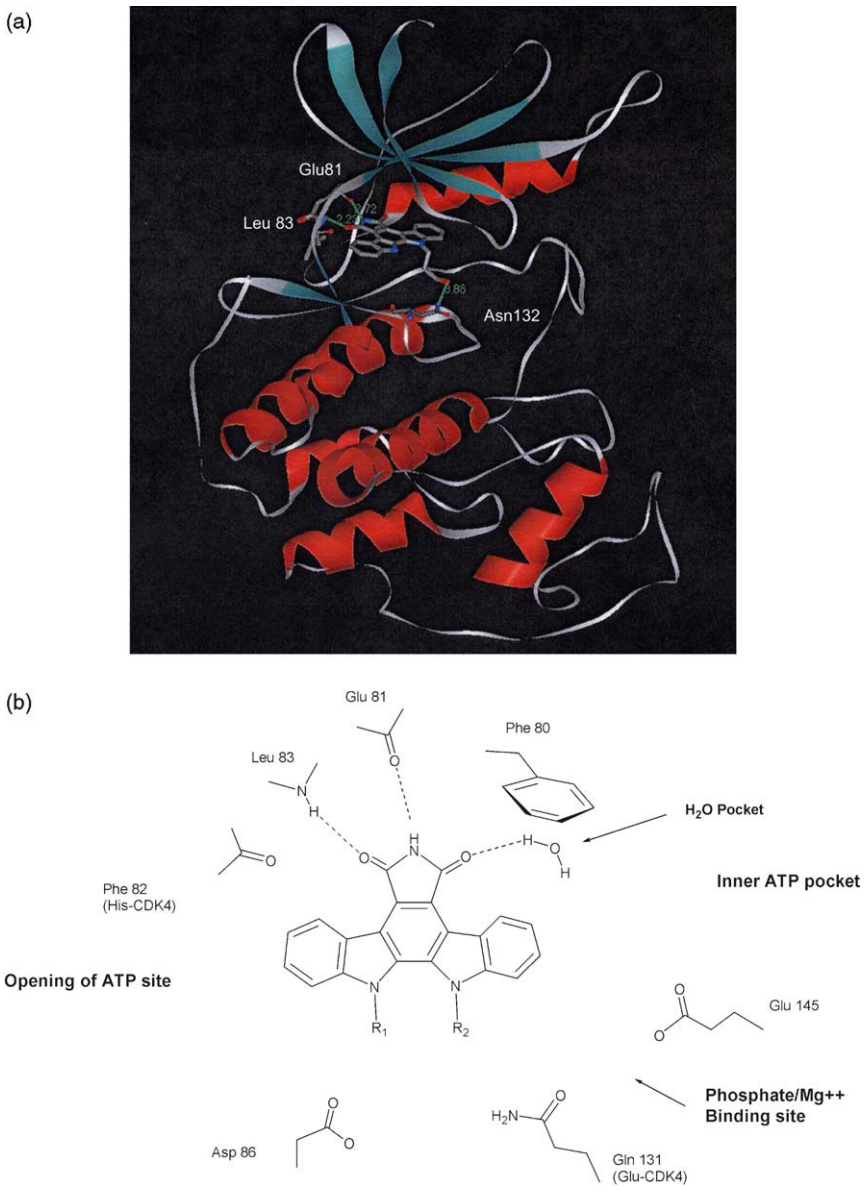


Figure 2. (a) The X-ray crystal structure of the human CDK2-4d complex; (b) Interaction of indolocarbazoles in ATP site of CDKs.

Table 3. Inhibition of Rb (Ser 780) phosphorylation with indolocarbazoles[a]

	Compd			
	4b	**4c**	**4e[b]**	**4h**
HCT-116 IC_{50} (μM)	0.78	1.7	0.52	0.47
% pRb inhibition @ IC_{50}	82	86	54	36
% pRb inhibition @ $2IC_{50}$	86	85	44	76
% pRb inhibition @ $3IC_{50}$	89	91	60	84

[a]Colon carcinoma cell (HCT-116) treated with compounds at 1×, 2× and 3× the antiproliferation IC_{50} concentration for 24 h.

in residues 82, 83 and 131 presumably contribute to the selectivity between CDK2 and CDK4 (Fig. 2b).

In summary, a comparison between bis-indolylmaleimides and indolocarbazoles as CDKs inhibitors is described. Indolocarbazoles showed greater potency towards CDK4 and CDK2 inhibition in a head to head comparison with their corresponding bis-indolylmaleimides. Both maleimides and carbazoles are capable of inhibiting cell growth in the human tumor cell lines HCT-116 and NCI H460. The specific G1 cell cycle arrest and selective inhibition of phosphorylation of serine 780 shown by the indolocarbazoles is consistent with the in vitro CDK4 inhibitory activity. Flow cytometry analysis showed that indolocarbazoles cause a G1 accumulation whereas bis-indolylmaleimides produce a G2/M arrest.

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