Pergamon

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Bioorganic & Medicinal Chemistry Letters 13 (2003) 2261–2267

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Novel, Potent and Selective Cyclin D1/CDK4 Inhibitors: Indolo[6,7-*a*]pyrrolo[3,4-*c*]carbazoles

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Received 10 February 2003; revised 2 May 2003; accepted 5 May 2003

Abstract—The synthesis and CDK inhibitory properties of a series of indolo[6,7-*a*]pyrrolo[3,4-*c*]carbazoles is reported. In addition to their potent CDK activity, the compounds display antiproliferative activity against two human cancer cell lines. These inhibitors also effect strong G1 arrest in these cell lines and inhibit Rb phosphorylation at Ser780 consistent with inhibition of cyclin D1/CDK4.

Cell cycle progression leading to cell division and proliferation is strictly controlled by the timed expression of various cyclins which associate with specific cyclin-dependent kinases (CDKs).[1,2] For example, passage through the G1 phase of the cell cycle is regulated by cyclin D1 and CDK4/6. These kinases regulate the release of the E2F family of transcription factors through hyperphosphorylation of the retinoblastoma tumor suppressor protein (Rb). Cyclin D1/CDK4/6 is in turn controlled by the p16 family of tumor suppressor genes. Aberrations of the Rb signalling pathway are frequent in human cancers.[3] Such aberrations include deletion or mutation of the Rb gene, mutations in p16 or CDK4, or overexpression or upregulation of cyclin D1 and/or CDK4. Another regulator of cyclin D1 is β-catenin and elevated levels of this protein are also associated with many cancers.[4] For these reasons, inhibitors of D1/CDK4,[2] particularly selective inhibitors,[5] have attracted much attention recently as potential therapeutic agents.

A variety of CDK inhibitors have been reported[2,5,6] including the indolo[2,3-*a*]carbazoles **1a**, **1b**, **2** and related structures (Fig. 1).[7] We have been interested in exploring novel analogues of these indolocarbazoles in which one indole moiety is replaced by different aryl groups.[6] Herein we report the activity of *indolo[6,7-a]*pyrrolo[3,4-*c*]carbazoles **3** as a new class of potent and selective inhibitors of cyclin D1/CDK4.

Indolocarbazoles **3** were prepared as shown in Scheme 1. Condensation of indole-7- or indole-3-acetamides (**6** and **9**) with indole-3- or 7-glyoxylates (**7** and **8**) according to the procedure reported by Faul[8] gave maleimides **10** that were cyclized photochemically[9] in the presence of DDQ or iodine to give **3**. Indole-7-acetamides **6** were prepared from commercially available indole-7-carboxaldehyde **4** via a modification[10c] of the Kurihara

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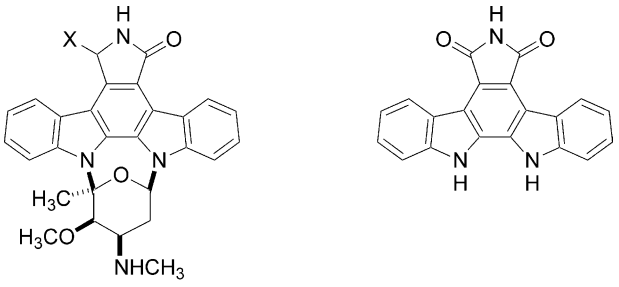
doi:10.1016/S0960-894X(03)00461-X



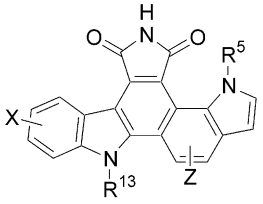


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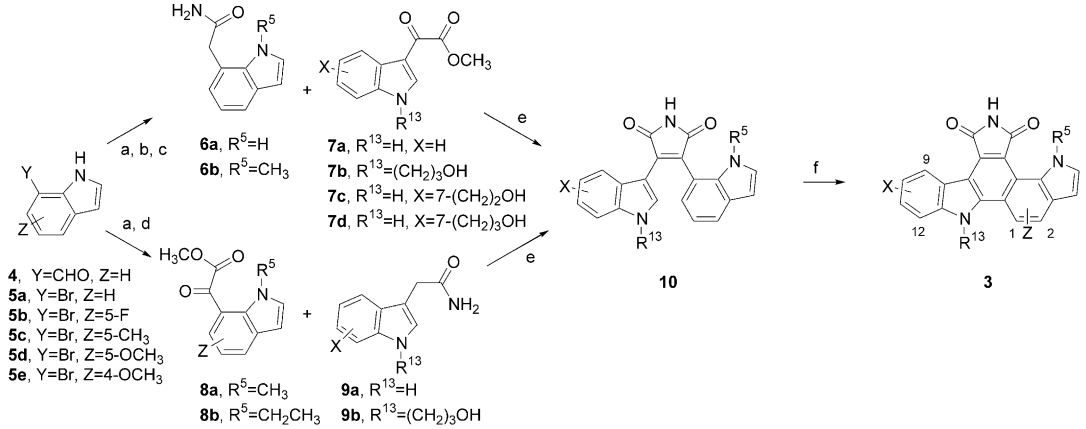
Figure 1. Chemical structures for (+)-Staurosporine (**1a**), UCN-01 (**1b**), arcyriaflavin A (**2**) and indolo[6,7-*a*]pyrrolo[3,4-*c*]carbazoles (**3**).

procedure,[10] and indole-7-glyoxylates **8** were produced from 7-bromoindoles **5a–e**. Bromoindole **5a** was commercially available and **5b–e** were produced from the corresponding 2-bromoanilines by the method of Sugasawa.[11] Indole-3-glyoxylates **7a** were prepared from the corresponding indoles by reaction with oxalyl chloride followed by treatment with NaOMe; indoleglyoxylate **7b** was prepared by alkylation of indole with $Br(CH_2)_3OTBDMS$ (NaH/DMF) followed by glyoxylation. The 7-(2-hydroxyethyl)indole precursor to **7c** was prepared via a Bartoli synthesis[12] using the TBDMS-ether of 2-nitrophenyl-ethanol. Indole-7-carboxaldehyde was converted into **7d** by the sequence (i) Wadworth–Emmons reaction with $(EtO)_2P(O)CH_2CO_2Et$ (KO*t*Bu/THF), (ii) hydrogenation (Pd/C, THF/MeOH), (iii) LAH reduction (THF) and (iv) TBDMSCl, imidazole (THF) to give the TBDMS-ether of 7-(3-hydroxypropyl)indole followed by glyoxylation as described above. *N*-(3-hydroxypropyl)-indole-3-acetamide **9b** was prepared by alkylation of indole-3-acetonitrile with $Br(CH_2)_3OAc$ (NaH/DMF) followed by hydrolysis with KOH/*t*-BuOH.

The kinase inhibitory activities of carbazoles **3** against cyclin D1/CDK4 and cyclin E/CDK2 were evaluated in enzymatic assays by measuring phosphorylation of Rb^{ING} and Rb^{21} according to standard protocols (Table 1).[13,14] Lower IC_{50}'s were consistently observed with the more physiologically relevant Rb^{21} substrate[13] compared to Rb^{ING}. Inhibition of PKA (histone) was also measured to preliminarily assess selectivity. In addition, effects on cell proliferation in vitro were determined in two human carcinoma cell lines, HCT-116 (colon) and NCI-460 (lung).[15]

The data in Table 1 reveals that the parent compound **3a** is a potent CDK inhibitor and displays antiproliferative activity. Methyl or ethyl groups are tolerated at N-5 (**3b**, **c**), but some loss in cellular potency is found with the latter; larger substituents at this position are significantly less active (>1 μM CDK4 IC_{50}, data not shown). Fluorine substitution at C-9 and C-10 gives compounds **3d** and **3h** with good enzymatic activity, but lacking antiproliferative properties. Significant decreases in enzymatic potency are observed when other substituents were introduced at C-9/C-10 (**3e–g**, **i–l**, respectively). The position tolerating the widest range of substituents in terms of CDK inhibition is C-11 (**3m–w**), and to a lesser extent C-12 (**3z–3cc**). However, a number of these compounds do not exhibit activity in cells, likely due to their poor aqueous solubility. Addition of hydroxyalkyl groups to C-12 or N-13 (**3bb–cc**, **3kk–oo**) gives potent inhibitors and cellular activity is retained provided that hydrogen is present at C-1. Kinetic analysis of a number of the inhibitors, **3v**, **3bb**, **3ll** revealed them to be reversible ATP-competitive inhibitors (data to be published elsewhere).

Compounds bearing substituents at C-1 and C-2 have also been examined (**3dd–3jj**). Good enzymatic and cellular activity is found for those incorporating a substituent at C-1 (**3dd–3jj**). However, activity is significantly lowered with C-1 and N-13 di-substitution (**3qq–3ss**). In the latter, one of the two substituents is likely forced out of plane due to steric interaction with



Scheme 1. (a) NaH, DMF, $(MeO)_2SO_2$ or EtI; (b) (i) $(EtO)_2P(O)CN$, [LiCN·THF], THF; (ii) *t*BuOH; (iii) SmI_2; (c) KOH, *t*BuOH; (d) *t*BuLi, THF, −78 °C, MeO_2CCO_2Me; (e) KO*t*Bu, DMF or THF; (f) hν, DDQ or I_2, dioxane or EtOAc.

the other making it difficult to fit into the narrow ATP binding pocket of the enzyme.

Although in most cases there appears to be a slight selectivity for CDK4 versus CDK2, the carbazoles **3** inhibit both kinases potently. However, they exhibit good selectivity with respect to PKA.

The aqueous solubility of indolocarbazoles **3** was poor; for example, aqueous solubility of **3v** at various pHs was <1 μg/mL. Data in Table 1 established that large substituents [e.g., $(CH_2)_3OH$] are well tolerated at N-13 and C-12. For further SAR, we chose to focus on introducing groups to these two positions to improve aqueous solubility of the platform. A summary of the aminoalkyl-substituted indolocarbazoles studied is shown in Figure 2.

Synthesis of the aminoalkyl-indolocarbazoles **11**/**12** is shown in Scheme 2. Conversion of (hydroxyalkyl) indolo-maleimides **15** into carbazoles **11**/**12** was effected in one of three ways. (1) The hydroxyalkyl side chain in **15** was converted into a bromoalkyl group followed by oxidative photocyclization to bromoalkylcarbazole **17**. Reaction of **17** with a variety of amines afforded **11**/**12**. (2) Alternatively, amine displacement on **16** to give aminoalkylmaleimides **18** could be effected prior to the photocyclization to **11**/**12**. (3) Finally, oxidative photocyclization of **15** produced hydroxyalkyl-carbazole **3** that could then be subjected to hydroxy to bromide exchange followed by amine displacement to yield **11**/**12**. Tactically, the first route (**15**→**16**→**17**→**11**/**12**) was favored due to the ease of handling **16**/**17** relative to the other intermediates (higher solubility in organic solvents and ease of purification). Similar transforma-

Table 1. Kinase inhibitory activity (IC_{50}, μM) against D1/CDK4, E/CDK2 and PKA, and antiproliferative activity (IC_{50}, μM) of indolocarbazoles **1**–**3**

Compd	R^5	R^{13}	X	Z	CDK4 (Rb^{ING})	CDK4 (Rb^{21})	CDK2 (Rb^{ING})	PKA	HCT-116	H460
1a					0.059	—[a]	—	—	—	—
2					0.140	0.163	0.897	—	0.85	0.59
3a	H	H	H	H	0.036	0.022	0.064	>2	1.44	1.43
3b	CH_3	H	H	H	0.047	0.022	0.075	>2	2.11	1.17
3c	CH_2CH_3	H	H	H	0.059	0.019	—	>2	4.52	>10
3d	CH_3	H	9-F	H	0.071	0.081	>2	>20	>2	>10
3e	CH_3	H	9-Br	H	0.171	0.136	2.86	>20	4.71	3.52
3f	CH_3	H	9-CH_2OH	H	0.155	0.216	>2	>20	>10	>10
3g	CH_3	H	9-OCH_3	H	>2	—	—	—	>10	>10
3h	CH_3	H	10-F	H	0.084	0.039	>0.2	>20	>10	>10
3i	CH_3	H	10-Br	H	>2	—	—	—	—	—
3j	CH_3	H	10-CF_3	H	>2	—	—	—	7.04	7.85
3k	CH_3	H	10-CH_3	H	0.393	0.407	1.07	4.06	2.87	3.35
3l	CH_3	H	10-OCH_3	H	0.519	0.680	>2	>20	>10	>10
3m	CH_3	H	11-F	H	0.038	0.011	0.009	>20	>10	>10
3n	CH_3	H	11-Br	H	0.038	<0.013	0.075	>20	1.08	0.72
3o	CH_3	H	11-CF_3	H	0.076	0.009	0.225	>20	>10	3.51
3p	CH_3	H	11-CN	H	0.029	0.011	0.010	>20	>10	>10
3q	CH_3	H	11-CH_3	H	0.048	0.018	0.320	>20	3.84	3.41
3r	CH_3	H	11-CH_2CH_3	H	0.101	0.022	0.360	>20	3.51	7.49
3s	H	H	11-OH	H	0.037	0.017	0.006	0.949	0.17	0.23
3t	CH_3	H	11-OH	H	0.033	0.009	—	2.35	0.67	1.08
3u	H	H	11-OCH_3	H	0.067	0.036	0.125	11.4	2.16	1.92
3v	CH_3	H	11-OCH_3	H	0.053	0.006	0.112	>20	1.80	1.09
3w	CH_3	H	11-OCH_2CH_3	H	0.044	0.010	0.324	>20	2.35	3.20
3x	H	H	11-OBn	H	0.187	0.378	—	—	>10	>10
3y	CH_3	H	11-OBn	H	0.123	0.025	0.328	3.29	6.52	>10
3z	CH_3	H	12-Br	H	0.093	—	—	—	17.1	13.8
3aa	CH_3	H	12-OCH_3	H	0.062	0.040	>2	>20	1.00	2.71
3bb	CH_3	H	12-$(CH_2)_2OH$	H	0.082	0.075	0.210	>2	1.13	—
3cc	CH_3	H	12-$(CH_2)_3OH$	H	0.168	0.040	0.558	>20	3.02	2.35
3dd	CH_3	H	H	1-CH_3	0.028	0.002	0.070	>20	1.98	2.26
3ee	CH_3	H	H	1-F	0.067	0.016	0.086	>20	3.49	2.90
3ff	CH_3	H	H	1-OCH_3	0.032	0.018	0.067	—	1.90	0.69
3gg	CH_3	H	H	2-OCH_3	0.054	—	0.035	13.2	1.13	0.71
3hh	CH_3	H	11-F	1-CH_3	0.050	0.006	0.023	>20	0.58	1.02
3ii	CH_3	H	11-OCH_3	1-CH_3	0.042	0.007	0.057	>20	1.43	1.30
3jj	CH_3	H	11-CF_3	1-CH_3	0.092	0.025	>2	>20	8.78	2.42
3kk	H	$(CH_2)_3OH$	H	H	0.066	0.012	0.008	>20	0.75	1.07
3ll	CH_3	$(CH_2)_3OH$	H	H	0.096	0.042	0.067	>2	1.36	1.13
3mm	CH_2CH_3	$(CH_2)_3OH$	H	H	0.134	0.090	0.400	5.31	1.20	1.77
3nn	CH_3	$(CH_2)_2OH$	11-OCH_3	H	0.047	0.024	0.101	1.62	4.07	1.32
3oo	CH_3	$(CH_2)_3OH$	11-OCH_3	H	0.054	0.012	0.113	1.04	1.62	0.50
3pp	CH_3	CH_3	H	1-CH_3	0.092	0.045	0.267	3.90	4.70	>10
3qq	CH_3	$(CH_2)_3OH$	H	1-CH_3	0.673	—	—	—	3.85	4.75
3rr	CH_3	$(CH_2)_4OH$	H	1-CH_3	>2	—	—	11.7	>10	>10
3ss	CH_3	$(CH_2)_3OH$	H	1-F	0.346	0.448	—	—	2.39	2.48

[a]Not tested.

tions employing 7-(ω-hydroxyalkyl)-indolomaleimides **19** afforded the C-12 substituted carbazoles **13**/**14** (Scheme 3).

Indolocarbazoles **11**–**14** were all potent inhibitors of D1/CDK4, with the C-12 substituted analogues demonstrating the highest activity, as indicated most strikingly by the Rb[21] data ($IC_{50}s \leq 69$ nM). Representative data is presented in Table 2. Kinetic analysis

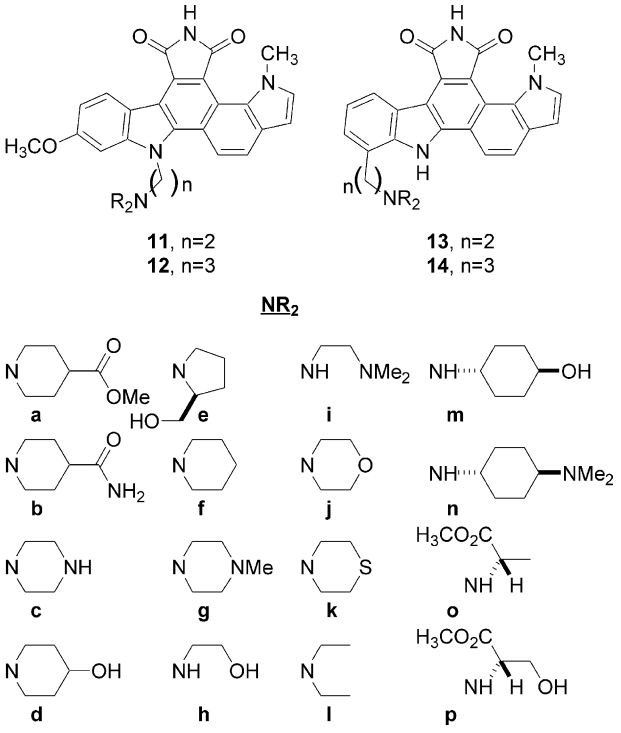
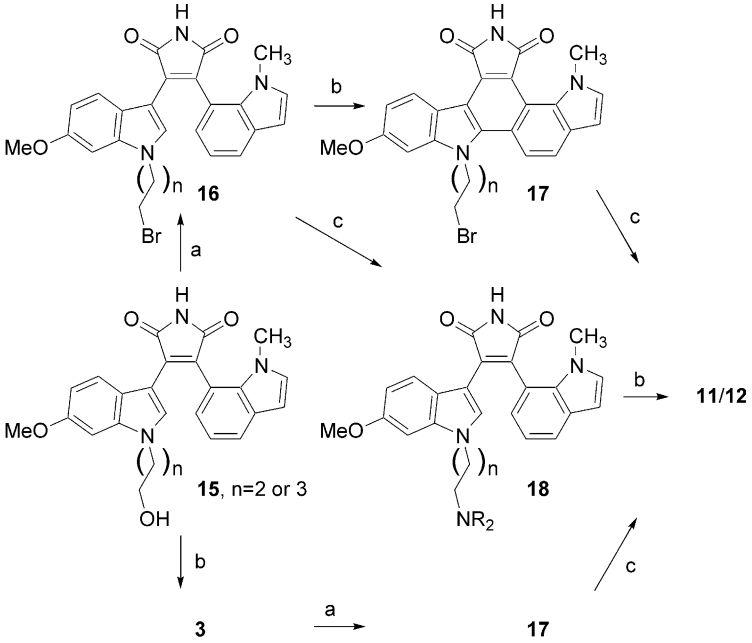


Figure 2. Aminoalkyl-substituted indolo[6,7-*a*]pyrrolo[3,4-*c*]carbazoles.

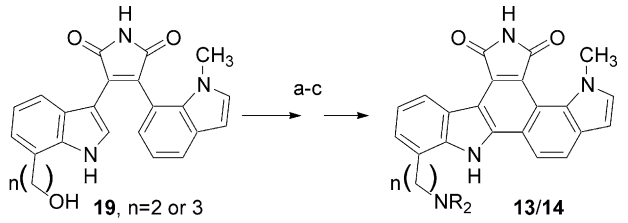
of **14m** again indicated a reversible ATP-competitive inhibitor (data to be published elsewhere). Compared to the indolo[6,7-*a*]pyrrolo[3,4-*c*]carbazoles lacking aminoalkyl side chains, **11**–**14** were significantly more selective with respect to E/CDK2, with many showing >20–40-fold selectivity and some >40-fold (**13a**, **12d**, **14l**). Interestingly, the scaffolds bearing amine **h** usually showed the greatest inhibition of CDK2. Regarding inhibition of other kinases, **11**–**14** displayed typically little activity for PKA. Similarly, most **11**–**12** exhibited only modest inhibition of CaMKII. In contrast, the C-12 substituted compounds **13** were more potent inhibitors of CaMKII and the homologous analogues **14** were somewhat less active against this kinase. However, considering the higher K_m of ATP for CDK4 relative to these other kinases (>10-fold),[16] the intracellular selectivity (mM ATP) is likely considerably higher than in vitro $IC_{50}s$ suggest. Compound **14m** was chosen for further scrutiny against a diverse set of kinases. $IC_{50}s$ against GSK-3β (>20 μM), MLK-7 (9.97 μM), TGF-β RII (>20 μM), p38α-MAPK (20.2 μM) and AKT-1 (5.48 μM) indicated comparatively little activity. Additional profiling against a wider range of both serine/threonine and tyrosine kinases (Table 3) showed little inhibition at inhibitor concentrations of 500 nM except for Blk and Lyn. For the latter, only modest inhibition was observed at 100 nM **14m**.

Most indolocarbazoles **3**, and **11**–**14** were also antiproliferative agents (Tables 1 and 2).[15] Cell cycle effects in HCT-116 cells of a number of these compounds were then examined by flow cytometry (Table 4),[17] and inhibition of Rb phosphorylation at the CDK4 specific Ser780 residue[18] was investigated by Western Blot analysis. These studies showed significant G1 cell cycle arrest and strong inhibition of Rb phosphorylation, consistent with inhibition of cyclin D1/CDK4.[14c]



Scheme 2. (a) PPh_3, CBr_4, DMF or CH_2Cl_2: THF; (b) hν, DDQ or I_2, dioxane or EtOAc; (c) NHR_2, DMF, 65 °C.

A primary driver for the SAR summarized in Figure 2 was to improve the aqueous solubility of the indolocarbazole platform. Several of these aminoalky-substituted indolocarbazoles were converted in situ to their methanesulfonate salts in D5W. Solubilities of >6 mg/mL were attained and these solutions were stable for more than one week at room temperature (Table 4). This compares very favorably with solubilities found with non-basic analogues (≤1 μg/mL).



Scheme 3. (a) PPh_3, CBr_4, DMF or CH_2Cl_2: THF; (b) hν, DDQ or I_2, dioxane or EtOAc; (c) NHR_2, DMF, 65 °C.

In summary, the discovery and SAR of a new series of potent CDK inhibitors based on the indolo[6,7-*a*]pyrrolo[3,4-*c*]carbazole scaffold is described. Simple derivatives are potent inhibitors of both cyclin D1/CDK4 and cyclin E/CDK2, but generally inactive or only modestly active against PKA. However, attachment of aminoalkyl substituents significantly improves the CDK4 inhibitory activity relative to CDK2 and dramatically increases aqueous solubility. In vitro characterization reveals that the antiproliferative activity exhibited by these agents is consistent with inhibition of cyclin D1/CDK4.

Table 2. Kinase inhibitory activity (IC_{50}, μM) against D1-CDK4, E-CDK2, CaMKII and PKA and antiproliferative activity (IC_{50}, μM) of representative indolocarbazoles **11-14**

Compd	*n*	NR2	CDK4 (Rb^{ING})	CDK4 (Rb^{21})	CDK2 (Rb^{ING})	PKA	CaMKII	HCT-116	H460
11e	2	**e**	0.084	0.023	0.942	>20	6.12	2.69	3.80
11f	2	**f**	0.135	0.033	0.928	>20	>20	3.74	2.47
11h	2	**h**	0.035	0.021	0.089	3.74	0.527	1.47	1.31
11m	2	**m**	0.063	0.023	0.902	18.0	1.17	0.89	1.05
12b	3	**b**	0.077	0.013	1.00	3.38	3.05	1.96	2.44
12d	3	**d**	0.048	0.004	2.10	2.81	3.13	1.26	1.13
12g	3	**g**	0.095	0.014	1.23	2.93	11.60	0.45	0.90
12h	3	**h**	0.072	0.028	0.432	3.15	0.732	1.55	1.28
12j	3	**j**	0.069	0.021	0.716	>20	>20	1.69	1.63
12k	3	**k**	0.190	0.047	1.26	>20	>20	2.02	2.11
13a	2	**a**	0.047	0.013	0.972	>20	0.081	>10	>10
13b	2	**b**	0.051	0.005	0.796	5.89	0.025	1.00	1.35
13c	2	**c**	0.049	0.015	2.03	12.9	0.301	1.04	1.11
13d	2	**d**	0.045	0.008	0.978	2.99	0.046	0.20	0.14
13e	2	**e**	0.039	0.023	0.685	4.58	0.090	0.42	0.21
13f	2	**f**	0.045	0.011	1.06	9.14	0.062	2.12	2.47
13h	2	**h**	0.033	0.010	0.265	6.12	0.095	0.17	0.33
13j	2	**j**	0.054	0.015	1.28	>20	0.177	1.63	1.91
13k	2	**k**	0.082	0.024	1.80	>20	0.403	3.41	3.41
13m	2	**m**	0.053	0.005	0.450	9.81	0.049	1.23	2.82
14g	3	**g**	0.052	0.035	1.67	16.6	0.382	1.38	1.48
14h	3	**h**	0.040	0.019	0.567	8.31	0.266	0.77	1.10
14j	3	**j**	0.056	0.043	1.33	>20	0.516	2.82	1.68
14l	3	**l**	0.034	0.024	1.50	14.1	0.174	0.95	0.37
14m	3	**m**	0.049	0.019	1.03	2.93	0.216	1.05	1.67

Table 3. Kinase Profiling of **14m** against 39 kinases; data is presented as activity (% control) in the presence of 100/500 nM of inhibitor[a,b]

Kinase	% Control	Kinase	% Control	Kinase	% Control
c-Raf	112/107	ROCK-II	90/62	CDK5/p35	94/62
MEK1	95/87	CK2	99/94	CK1	98/89
MAPK2	103/90	Lck	98/60	CSK	104/116
MKK6	100/98	PRAK	86/50	IKKβ	120/106
SAPK3	107/109	Fyn	92/74	Lyn	78/18
MSK1	92/90	ZAP-70	134/107	PKCθ	82/58
MKK4	91/84	CHK2	84/53	Syk	94/68
MKK7β	86/70	PRK2	97/88	p70S6K	93/85
JNK1α1	99/88	PKCβII	96/59	CHK1	86/70
PDK1	104/65	PKCγ	105/84	AMPK	98/95
SGK	94/84	Blk	74/14	JNK3	105/97
PKCα	100/84	CaMKIV	108/102	cSRC	100/91
MAPKAP-K2	94/104	Cyclin E/CDK3	89/63	Cyclin A/CDK2	83/42

[a][ATP] at K_m where known or 10 μM.
[b]Mean of duplicate assays.

Table 4. G1 arrest and inhibition of Rb (Ser780) phosphorylation in HCT-116 cells, and aqueous solubility of selected indolocarbzoles **3**, **11**–**14**

Compd	G1 Arrest[a] (fold increase over control)		% Inhibition of Rb Phosphorylation[a] (Ser780)		Solubility[b]
	@1×IC_{50}	@3×IC_{50}	@1×IC_{50}	@3×IC_{50}	(mg/mL)
3b	1.4	2.5[d]	63	97	—[c]
3t	2.2	—	37	95	—
3v	2.6	—	90	97	—
3bb	1.4	2.7	60	92	—
3ff	1.8	2.1	56	77	—
3gg	1.2	2.2	71	86	—
3ii	2.0	2.2	83	95	—
3oo	2.4	—	66	95	—
11h	3.1	—	71	92	6.0
11m	1.8	2.0	67	95	7.8
12b	1.9	2.5[e]	58	67	—
12h	1.8	2.7	57	78	8.6
13b	1.9	2.2	52	55	—
13d.TFA	1.8	—	68	69	—
13e.TFA	1.5	—	65	73	—
13f	1.8	2.1	69	78	8.5
13j	1.5	2.2	57	88	8.7
13j.HCl	1.2	2.3	69	80	—
13m	2.0	—	78	87	—
14m	2.9	3.4	45	83	8.9

[a]After 24 h incubation.
[b]As MsOH salts formed in situ in D5W after 1 week.
[c]Not reported.
[d]At 2.4×IC_{50}.
[e]At 2.6×IC_{50}.

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