Full Paper

Synthesis and Biological Evaluation of 7-Azaisoindigo Derivatives

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A series of novel 7-azaisoindigo derivatives **3**–**14** were designed, synthesized, and structurally characterized by IR, ^{1}H-NMR, ^{13}C-NMR, mass spectra, and elemental analyses. Their antiproliferative activities were evaluated in a hormone-independent prostate cancer cell line DU145. Among them, compounds **8**, **9**, **14** showed the highest activities. Our study also showed that compounds **7**, **11**, **12** exhibited higher inhibitory activities on CDK2/cyclin A than that of the positive control meisoindigo. Western blot analysis on DU145 cells treated with compounds **7** and **9** demonstrated that 7-azaisoindigo derivatives could decrease the level of CDK2 activity (phosphorylation) and the expression of cyclin D1, and increase the expression of endogenous cyclin-dependent inhibitor p27.

Keywords: Antitumor activity / 7-Azaisoindigo / CDK2/cyclin A assay / Synthesis

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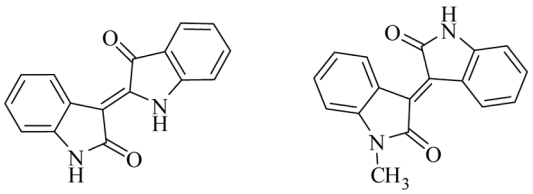
Introduction

Traditional medicines, in particular traditional Chinese medicines (TCM), represent a valuable source of developing potential new antineoplastic drugs. One good example is the successful development of the anti-chronic myelocytic leukemic (CML) drug indirubin from the TCM recipe Danggui Longhui Wan [1–4]. Indigo and its red isomer, indirubin **I** (Fig. 1), were isolated from Qingdai (an active ingredient of the recipe) as active components against CML [5–10] and have been approved for clinical trials against chronic myelocytic and chronic granulocytic leukemia [11–14]. Indirubin and its derivatives have clearly antiproliferative effects. Meijer and co-workers reported that indirubin derivatives selectively inhibit cyclin-dependent kinases (CDKs), a family of key cell-cycle regulators [15], through competing with ATP for binding to the catalytic site of the kinase. Indirubins are also strong inhibitors of an evolutionarily related kinase, glycogen synthase kinase-3β (GSK-3β) [16–18]. However, indirubin shows poor solubility, thus poor bioavailability and gastrointestinal side effects. Developing new indirubin derivatives aimed at improving pharmacological properties and reducing toxicities have been extensively carried out by a group of Chinese scientists [19–21].



I **II**

Figure 1. Structures of indirubin **I** and methylisoindigotin **II**.

Consequently, methylisoindigotin **II** (Fig. 1), or abbreviated as meisoindigo, a bis-indole compound, showed a higher antileukemic activity than indirubin and had fewer clinical side effects [19, 22–25]. Meisoindigo strongly inhibits DNA biosynthesis and microtubule assembly in tumor cells [24, 26]. It also induces the leuke-

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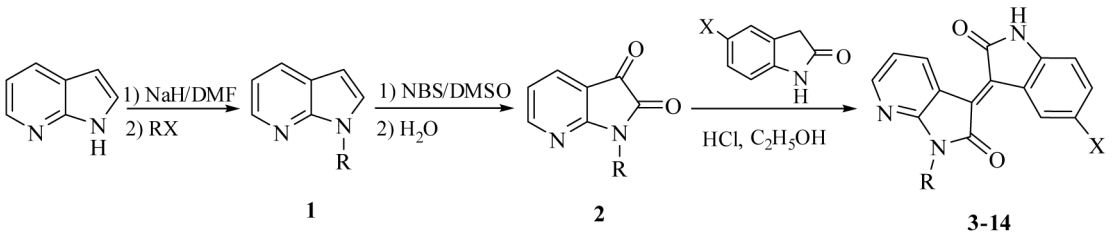
Abbreviation: rotational nuclear Overhauser effect spectroscopy (ROESY)



Scheme 1. Synthetic route of 1-alkyl-5′-halo-7-azaisoindigo derivatives.

Table 1. Synthesized 7-azaisoindigo derivatives **3–14**.

Compound	R	X	Yield (%)	Compound	R	X	Yield (%)
3	CH_3	F	72.6	**9**	*i*-C_3H_7	Br	79.7
4	*n*-C_4H_9	H	80.2	**10**	C_2H_5	H	78.5
5	*i*-C_3H_7	Cl	72.8	**11**	C_2H_5	Cl	73.8
6	C_2H_5	Br	71.8	**12**	*n*-C_4H_9	F	74.9
7	C_2H_5	F	74.1	**13**	*n*-C_4H_9	Br	81.4
8	*n*-C_4H_9	Cl	78.6	**14**	*i*-C_3H_7	H	72.8

mic cell differentiation by decreasing the *c-myb* expression [27]. A recent study indicated that the anti-angiogenesis effect of meisoindigo may also contribute to its antileukemic effect [28].

A more recent study indicates that the presence of a pyridine N-atom may reinforce the DNA- and/or enzyme-binding properties [29] of those nitrogenous aromatic compounds. Therefore, we designed and synthesized a series of 7-azaisoindigo derivatives **3–14**, in which an indole ring is coupled with a 7-azaindole ring at the 3,3′-position, in order to get more potent and selective antitumor agents. 7-Azaindole derivatives can inhibit the proliferation of tumor cells [30, 31]. The 7-azaindole moiety in the molecules may increase their antitumor activity. In this manuscript, we describe the methods to synthesize these compounds and the results of their activities on inhibitions of proliferation and CDK signaling in a hormone-independent prostate cancer cell line.

Results and discussion

Synthesis

All 7-azaisoindigo derivatives **3–14** were obtained as described in the Scheme 1. The structures of compounds **3–14** are indicated in Table 1.

The key intermediate, 1-alkyl-7-azaindole-2,3-dione **2**, was prepared by alkylation of 7-azaindole and subsequent oxidation with *N*-bromosuccinimide (NBS) in DMSO [32]. The required diversely substituted indolin-2-one derivatives were obtained commercially. Condensation of compound **2** in the presence of substituted indolin-2-one derivatives produced the corresponding 1-alkyl-5′-substituted-7-azaisoindigos **3–14** in 71–81% yield (Table 1).

Compounds **3–14** were structurally characterized by their IR, ^{1}H-NMR, ^{13}C-NMR, ESI-MS spectra, and elemental analysis. 7-Azaisoindigos might be formed by coupling an indole ring with a 7-azaindole ring at the 3,3′-position in (*E*)-configuration. Although there is no H-atom attached to C-atoms of the double bond, we could elucidate their configuration through ROESY (Rotational nuclear Overhauser Effect Spectroscopy) method.

The ROESY results were applicable for the determination of the *E*-/*Z*-configuration of compound **9** (Fig. 2). The individual structures of the *E*- and *Z*-isomers were generated by the sybyl program. The calculated distances between H-4 and H-4′ was >0.5 nm for the *E*-isomer, which was consistent with the result that no ROESY correlation was observed between H-4 and H-4′, while for the *Z*-isomer, the calculated distance between H-4 and H-4′ was 0.23 nm, which was considered to have a strong ROE correlation.

Antitumor activities

The antitumor activities of compounds **3–14** were tested in cell line DU145 (hormone-independent prostatic carcinoma) *in vitro* by the standard MTT method using meisoindigo **II** as a positive control. The results expressed as IC_{50} are summarized in Table 2.

As shown in Table 2, most of the evaluated compounds showed potent inhibitory effects on the growth of DU145 cells. Among them, compounds **8**, **9**, and **14** which pos-

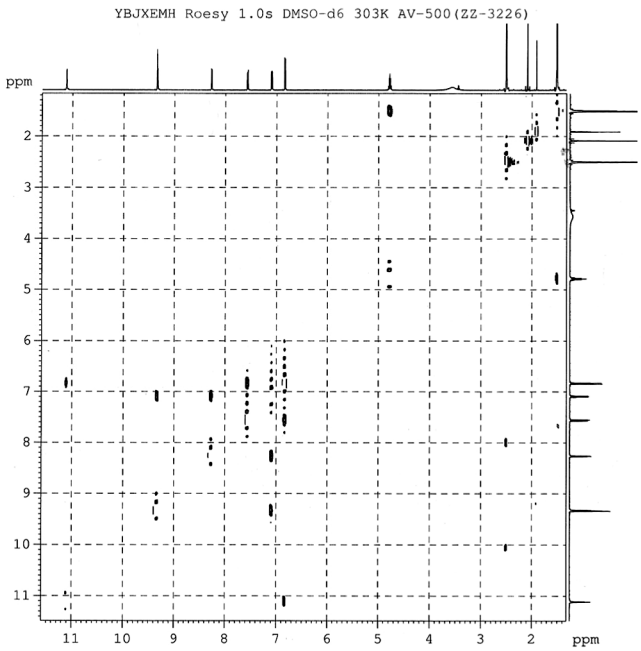


Figure 2. ROESY (Rotational nuclear Overhauser effect spectroscopy) spectrum of compound **9**.

Table 2. Antitumor activities of the compounds **3–14** and **II**.

Compound	IC_{50} (μmol/L)	Compound	IC_{50} (μmol/L)
3	43	**9**	8.3
4	14	**10**	10.5
5	10	**11**	14.0
6	14	**12**	20.7
7	17.5	**13**	15
8	9.0	**14**	9.1
Meisoindigo (II)			17.4

sess *i*Pr or Bu residues at *N*(1), showed the strongest growth inhibition on DU145 cells, while compounds **3** and **12** containing a F-atom at the 5′-position were less potent than meisoindigo **II**. Other compounds showed similar activities as the reference compound.

Inhibitory effects on CDK2/cyclin A

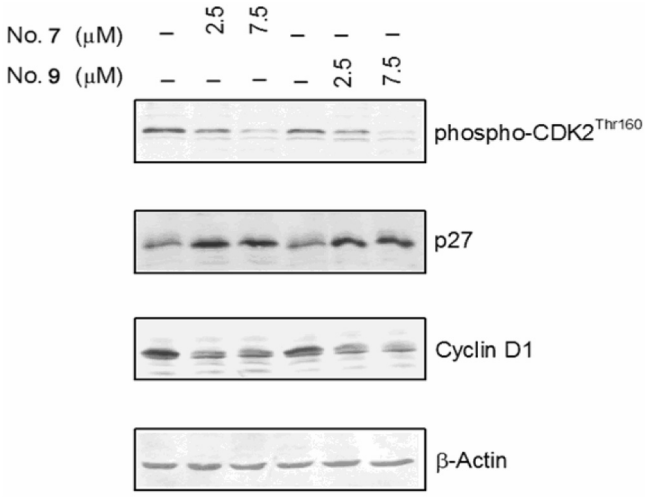
To evaluate whether these indirubin derivatives are also CDK inhibitors, we examined their activity on CDK2/cyclin A and meisoindigo **II** served as a positive control at a final concentration of 10^{-5} mol/L. The results are listed in Table 3.

We found that compounds **7**, **11**, **12** exhibited the strongest potency of inhibition of recombinant human CDK2/cyclin A kinase (percent inhibition >50%) at a final concentration of 10^{-5} mol/L *in vitro*, which was found to be more potent than meisoindigo. Our findings suggest

Table 3. Inhibition of the compounds **3–14** and **II** on CDK2/cylin A.

Compound	Inhibition (%)§	IC_{50} (μmol/L)	Compound	Inhibition (%)§	IC_{50} (μmol/L)
3	12 ± 1	ND&	**10**	16 ± 3	ND
4	12 ± 2	ND	**11**	78 ± 7.5	27.0
5	12 ± 1	ND	**12**	81 ± 9	6.2
6	13 ± 1.5	ND	**13**	11 ± 1	ND
7	75 ± 5	76.0	**14**	11 ± 0.6	ND
8	11 ± 0.5	ND			
9	11 ± 1	ND	**II**	66 ± 7	89.0

§ Determined at a final concentration of 10^{-5} mol/L; & ND: not determined.



DU145 cells were exposed to compounds **7** or **9** for 24 h. Cellular proteins were extracted and immunoblotted using indicated antibodies where β-actin served as a loading control.

Figure 3. Western blotting analysis of protein levels of CDK2/cyclin D1 and p27.

that an appropriate length of the alkyl chain at the *N*(1)-position (*e.g.* Et) and a F-atom at the 5′-position were essential to the CDK2/cyclin A inhibition for these 7-azaisoindigo derivatives.

Effects on CDK2/cyclin D1 and p27 protein expressions in DU145 cells

To further investigate whether 7-azaisoindigo derivatives affect CDK activity in human tumor cells, we selected CDK2/cyclin D1 kinase as a representative target. We found that the level of phosphorylated CDK2 kinase was significantly reduced and expression of cyclin D1 was markedly down-regulated in a concentration-dependent manner when DU145 cells were exposed to compounds **7** and **9** for 24 h. Interestingly, those compounds up-regulated expression of endogenous CDK inhibitor p27 under the same experimental conditions (Fig. 3). The induction

of 7-azaisoindigo derivatives on expression of p27 is probably through their ability to activate the AhR receptor pathway as was reported for indirubins previously [33]. These observations paralleled the growth inhibition of the malignant cells.

Conclusion

In summary, we have synthesized a series of novel 7-azaindigo derivatives **3–14**. To evaluate the potential antitumor activity of these compounds, we examined their activities on inhibition of cell proliferation and CDK signaling in DU145 human prostate cancer cells. We found that most of the compounds showed a potent anticancer activity. Among them, compounds **8**, **9**, and **14** displayed a better activity than that of the positive control meisoindigo. Compounds **7**, **11**, and **12** significantly inhibited the activity of CDK2/cyclin A which was found to be stronger than that of the positive control agent meisoindigo in DU145 cells. It was also found that these compounds inhibited the activity of CDK2, down-regulated expression of cyclin D1, and up-regulated endogenous CDK inhibitor p27. Based on the above observation, we found that compound **11** was the most promising compound. Thus, 7-azaisoindigo derivatives are novel anticancer indirubin derivatives worth to be further investigated.

Experimental

General

All melting points were measured on a Büchi Melting Point B-540 apparatus (Büchi Labortechnik, Flawil, Switzerland) and were uncorrected. Mass spectra (MS) were taken in ESI model on Agilent 1100 LC-MSD (Agilent, Palo Alto, CA, USA). Proton (^{1}H) nuclear magnetic resonance spectroscopy was performed using Brucker ARX-300 (Bruker, Fällanden, Switzerland), operating at 300 MHz with TMS as an internal standard. The chemical shifts were reported in ppm (δ) and coupling constants (*J*) values were given in Hertz (Hz). Signal multiplicities were represented by: s (singlet), d (doublet), dd (double doublet), t (triplet), dt (triple triplet), m (multiplet). ^{13}C-NMR (125 MHz) spectra were recorded with Brucker AV-500 NMR spectrometer (Bruker). The IR spectra were performed on a FTIR8400S (Shimadzu, Kyoto, Japan) in KBr pellets; the frequencies were expressed in cm^{-1}. Elemental analysis was performed by an Elementar Vario EL III instrument (Heraeus GmbH, Hanau, Germany) for C, H, and N and the results were within ± 0.5% of the theoretical values. Unless noted otherwise, all solvents and reagents were commercially available and used without further purification.

*General procedure for the synthesis of compounds **3–14***

To a solution of 1-alkyl-7-azaindole-2,3-dione (2.47 mmol) and appropriately substituted indolin-2-one derivatives (2.47 mmol) in ethanol (15 mL), several drops of hydrochloric acid was added until pH = 2–3. The mixture was stirred at 70–75°C for 3 h. The reaction mixture was then allowed to cool down. The precipitate was formed, filtered, and washed by glacial acetic acid, water, and ethanol respectively to obtain compounds **3–14**. The compounds were further purified by recrystallization in glacial acetic acid to yield brownish red colored crystalline powders.

*(E)-3-(5-Fluoro-2-oxoindolin-3-ylidene)-1-methyl-1H-pyrrolo[2,3-b]pyridin-2(3H)-one **3***

Yield: 72.6%; brownish red crystals; m.p.: 291–293°C; IR (KBr): 3413, 3268, 3130, 1716, 1681, 1592, 1471, 1400, 1344, 1303, 1255, 1101, 1052, 790, 605, 555; ^{1}H-NMR (DMSO-d_6) δ: 3.63 (s, 3H, N-CH$_3$), 6.85 (d, 1H, *J* = 8.76 Hz, 7′-H), 7.11 (dd, 1H, *J* = 5.13, 7.89 Hz, 5-H), 7.26 (dd, 1H, *J* = 2.78, 8.76 Hz, 6′-H), 8.27 (dd, 1H, *J* = 1.59, 5.13 Hz, 6-H), 9.00 (dd, 1H, *J* = 2.78 Hz, 4′-H), 9.34 (dd, 1H, *J* = 1.59, 7.89 Hz, 4-H), 11.01(s, 1H, N′-H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 24.91 (NCH$_3$), 110.08 (C$_{7'}$), 115.57 (C$_{3a}$), 116.01 (C$_5$), 117.99 (C$_{3'a}$), 119.36 (C$_3$), 121.89 (C$_{4'}$), 130.78 (C$_{3'}$), 134.35 (C$_{6'}$), 136.42 (C$_4$), 140.82 (C$_{7'a}$), 149.78 (C$_6$), 156.05 (C$_{5'}$), 157.88 (C$_{1a}$), 167.11 (C$_2$), 168.73 (C$_{2'}$); ESI-MS *m*/*z*: 296.1 [M$^+$ + H]. Anal. calcd. for C$_{16}$H$_{10}$FN$_3$O$_2$ (295.27): C, 65.08; H, 3.41; N, 14.23. Found: C, 64.89; H, 3.49; N, 14.37.

*(E)-1-Butyl-3-(2-oxoindolin-3-ylidene)-1H-pyrrolo[2,3-b]pyridin-2(3H)-one **4***

Yield: 80.2%; brownish red crystals; m.p.: 215–216°C; IR (KBr): 3430, 3137, 2958, 2485, 1708, 1637, 1461, 1326, 1211, 1103, 796, 750, 661, 597; ^{1}H-NMR (DMSO-d_6) δ: 0.92 (t, 3H, CH$_3$), 1.33 (m, 2H, CH$_2$), 1.67 (m, 2H, CH$_2$), 3.82 (t, 2H, N-CH$_2$), 6.88 (d, 1H, *J* = 7.68 Hz, 7-H), 7.02 (dt, 1H, *J* = 1.16, 8.08 Hz, 5′-H), 7.10 (dd, 1H, *J* = 5.10, 7.86 Hz, 5-H), 7.38 (dt, 1H, *J* = 1.16, 7.68 Hz, 6′-H), 8.25 (dd, 1H, *J* = 1.57, 5.10 Hz, 6-H), 9.12 (d, 1H, *J* = 8.08 Hz, 4′-H), 9.33 (dd, 1H, *J* = 1.57, 7.86 Hz, 4-H), 11.02 (s, 1H, N′-H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 13.49 (CH$_3$), 19.54, 29.15 (CH$_2$), 38.27 (NCH$_2$), 109.70 (C$_{7'}$), 115.66 (C$_{5'}$), 117.89 (C$_{3a}$), 121.27 (C$_5$), 121.33 (C$_{3'a}$), 129.49 (C$_3$), 129.70 (C$_{4'}$), 133.39 (C$_{3'}$), 135.14 (C$_{6'}$), 136.23 (C$_4$), 144.67 (C$_{7'a}$), 149.45 (C$_6$), 156.62 (C$_{1a}$), 166.88 (C$_2$), 168.80 (C$_{2'}$); ESI-MS *m*/*z*: 320.1 [M$^+$ + H]. Anal. calcd. for C$_{19}$H$_{17}$N$_3$O$_2$ (319.3): C, 71.46; H, 5.37; N, 13.16. Found: C, 71.20; H, 5.42; N, 12.82.

*(E)-3-(5-Chloro-2-oxoindolin-3-ylidene)-1-isopropyl-1H-pyrrolo[2,3-b]pyridine-2(3H)-one **5***

Yield: 72.8%; brownish red crystals; m.p.: 270–271°C; IR (KBr): 3434, 3180, 1701, 1685, 1618, 1587, 1436, 1398, 1359, 1263, 1099, 1002, 810, 765, 615; ^{1}H-NMR (DMSO-d_6) δ: 1.51 (d, 6H, 2 CH$_3$), 4.78 (m, 1H, N-CH), 6.87 (d, 1H, *J* = 8.32 Hz, 7′-H), 7.09 (dd, 1H, *J* = 5.09, 7.90 Hz, 5-H), 7.45 (dd, 1H, *J* = 2.22, 8.32 Hz, 6′-H), 8.27 (dd, 1H, *J* = 1.52, 5.09 Hz, 6-H), 9.21 (d, 1H, *J* = 2.22 Hz, 4′-H), 9.34 (dd, 1H, *J* =1.52, 7.90 Hz, 4-H), 11.96 (s, 1H, N′-H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 19.00 (2 CH$_3$), 43.10 (NCH), 109.74 (C$_{7'}$), 115.71 (C$_{5'}$), 117.61 (C$_{3a}$), 121.31 (C$_5$), 121.37 (C$_{3'a}$), 129.76 (C$_3$), 129.85 (C$_{4'}$), 133.40 (C$_{3'}$), 134.99 (C$_{6'}$), 136.26 (C$_4$), 144.71 (C$_{7'a}$), 149.31 (C$_6$), 156.72 (C$_{1a}$), 166.63 (C$_2$), 168.90 (C$_{2'}$); ESI-MS *m*/*z*: 340.1 [M$^+$ + H] (Cl = 35), 342.1 [M$^+$ + H] (Cl = 37). Anal. calcd. for C$_{18}$H$_{14}$ClN$_3$O$_2$ (339.08): C, 63.63; H, 4.15; N, 12.37. Found: C, 63.46; H, 4.21; N, 12.16.

(E)-3-(5-Bromo-2-oxoindolin-3-ylidene)-1-ethyl-1H-pyrrolo[2,3-b]pyridin-2(3H)-one ***6***

Yield: 71.8%; brownish red crystals; m.p.: 293–295°C; IR (KBr): 3434, 3197, 3131, 1693, 1618, 1589, 1452, 1359, 1315, 1241, 1103, 896, 811, 611; ^{1}H-NMR (DMSO-d_6) δ: 1.24 (t, 3H, CH_3), 3.86 (q, 2H, N-CH_2), 6.83 (d, 1H, *J* = 8.29 Hz, 7′-H), 7.11 (dd, 1H, *J* = 5.12, 7.89 Hz, 5-H), 7.57 (dd, 1H, *J* = 2.06, 8.29 Hz, 6′-H), 8.28 (dd, 1H, *J* = 1.59, 5.12 Hz, 6-H), 9.33 (dd, 1H, *J* = 1.59, 7.89 Hz, 4-H), 9.37 (d, 1H, *J* = 2.06 Hz, 4′-H), 11.16 (s, 1H, N′-H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 13.51 (CH_3), 39.57 (NCH_2), 111.54 ($C_{7'}$), 112.89 ($C_{5'}$), 115.50 (C_{3a}), 117.95 (C_5), 122.96 ($C_{3'a}$), 130.97 (C_3), 131.72 ($C_{4'}$), 133.62 ($C_{3'}$), 135.33 ($C_{6'}$), 136.61 (C_4), 143.65 ($C_{7'a}$), 149.91 (C_6), 156.94 (C_{1a}), 166.93 (C_2), 168.41 ($C_{2'}$); ESI-MS *m*/*z*: 368.1 [M^- − H] (Br = 79), 370.1 [M^- − H] (Br = 81). Anal. calcd. for $C_{17}H_{12}BrN_3O_2$ (369.01): C, 55.15; H, 3.27; N, 11.35. Found: C, 55.27; H, 3.33; N, 11.28.

(E)-1-Ethyl-3-(5-fluoro-2-oxoindolin-3-ylidene)-1H-pyrrolo[2,3-b]pyridin-2(3H)-one ***7***

Yield: 74.1%; brownish red crystals; m.p.: 260–262°C; IR (KBr): 3423, 3193, 3133, 1702, 1637, 1591, 1467, 1361, 1311, 1257, 1201, 1103, 935, 811, 790, 765, 613; ^{1}H-NMR (DMSO-d_6) δ: 1.24 (t, 3H, CH_3), 3.84 (q, 2H, N-CH_2), 6.82 (d, 1H, *J* = 8.57 Hz, 7′-H), 7.09 (dd, 1H, *J* = 5.12, 7.88 Hz, 5-H), 7.23 (dd, 1H, *J* = 2.72, 8.57 Hz, 6′-H), 8.25 (dd, 1H, *J* = 1.57, 5.12 Hz, 6-H), 8.97 (d, 1H, *J* = 2.72 Hz, 4′-H), 9.33 (dd, 1H, *J* = 1.57, 7.88 Hz, 4-H), 10.99 (s, 1H, N′-H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 12.43 (CH_3), 33.37 (NCH_2), 110.04 ($C_{7'}$), 115.48 (C_{3a}), 116.04 (C_5), 117.88 ($C_{3'a}$), 119.29 (C_3), 121.80 ($C_{4'}$), 130.65 ($C_{3'}$), 134.31 ($C_{6'}$), 136.56 (C_4), 140.80 ($C_{7'a}$), 149.72 (C_6), 156.08 ($C_{5'}$), 156.89 (C_{1a}), 166.60 (C_2), 168.69 ($C_{2'}$); ESI-MS *m*/*z*: 310.1 [M^+ + H]. Anal. calcd. for $C_{17}H_{12}FN_3O_2$ (309.09): C, 66.02; H, 3.91; N, 13.59. Found: C, 65.95; H, 3.97; N, 13.27.

(E)-1-Butyl-3-(5-chloro-2-oxoindolin-3-ylidene)-1H-pyrrolo[2,3-b]pyridin-2(3H)-one ***8***

Yield: 78.6%; brownish red crystals; m.p.: 270–273°C; IR (KBr): 3432, 3310, 2956, 1704, 1618, 1589, 1456, 1400, 1384, 1313, 1101, 979, 817, 595; ^{1}H-NMR (DMSO-d_6) δ: 0.92 (t, 3H, CH_3), 1.35 (m, 2H, CH_2), 1.66 (m, 2H, CH_2), 3.82 (t, 2H, N-CH_2), 6.88 (d, 1H, *J* = 8.37 Hz, 7′-H), 7.11 (dd, 1H, *J* = 5.10, 7.89 Hz, 5-H), 7.45 (dd, 1H, *J* = 2.22, 8.37 Hz, 6′-H), 8.27 (dd, 1H, *J* = 1.59, 5.10 Hz, 6-H), 9.22 (d, 1H, *J* = 2.22 Hz, 4′-H), 9.33 (dd, 1H, *J* = 1.59, 7.89 Hz, 4-H), 11.14 (s, 1H, N′-H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 13.48 (CH_3), 19.55, 29.10 (CH_2), 38.34 (NCH_2), 110.95 ($C_{7'}$), 115.46 ($C_{5'}$), 118.03 (C_{3a}), 122.45 (C_5), 125.24 ($C_{3'a}$), 129.01 (C_3), 130.91 ($C_{4'}$), 132.51 ($C_{3'}$), 133.78 ($C_{6'}$), 136.68 (C_4), 143.31 ($C_{7'a}$), 149.95 (C_6), 156.90 (C_{1a}), 166.92 (C_2), 168.48 ($C_{2'}$); ESI-MS *m*/*z*: 354.1 [M^+ + H] (Cl = 35), 356.1 [M^+ + H] (Cl = 37). Anal. calcd. for $C_{19}H_{16}ClN_3O_2$ (353.8): C, 64.50; H, 4.56; N, 11.88. Found: C, 63.88; H, 4.57; N, 11.37.

(E)-3-(5-Bromo-2-oxoindolin-3-ylidene)-1-isopropyl-1H-pyrrolo[2,3-b]pyridin-2(3H)-one ***9***

Yield: 79.7%; brownish red crystals; m.p.: 264–265°C; IR (KBr): 3475, 3415, 3197, 1718, 1697, 1664, 1616, 1583, 1438, 1307, 1106, 783, 611, 541; ^{1}H-NMR (DMSO-d_6) δ: 1.51 (d, 6H, 2 CH_3), 4.78 (m, 1H, N-CH), 6.82 (d, 1H, *J* = 8.31 Hz, 7′-H), 7.08 (dd, 1H, *J* = 5.10, 7.92 Hz, 5-H), 7.55 (dd, 1H, *J* = 2.04, 8.31 Hz, 6′-H), 8.26 (dd, 1H, *J* = 1.60, 5.10 Hz, 6-H), 9.31 (d, 1H, *J* = 2.04 Hz, 4′-H), 9.33 (d, 1H, *J* = 1.60 Hz, 4-H), 10.99 (s, 1H, N′-H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 18.99 (2 CH_3), 43.22 (NCH), 111.50 ($C_{7'}$), 112.90 ($C_{5'}$), 115.49 (C_{3a}), 117.71 (C_5), 122.97 ($C_{3'a}$), 131.29 (C_3), 131.75 ($C_{4'}$), 133.44 ($C_{3'}$), 135.26 ($C_{6'}$), 136.62 (C_4), 143.62 ($C_{7'a}$), 149.75 (C_6), 156.99 (C_{1a}), 166.60 (C_2), 168.40 ($C_{2'}$); ESI-MS *m*/*z*: 382.1 [M^- − H] (Br = 79), 384.1 [M^- − H] (Br = 81). Anal. calcd. for $C_{18}H_{14}BrN_3O_2$ (383.03): C, 56.27; H, 3.67; N, 10.94. Found: C, 56.18; H, 3.73; N, 10.89.

(E)-1-Ethyl-3-(2-oxoindolin-3-ylidene)-1H-pyrrolo[2,3-b]pyridin-2(3H)-one ***10***

Yield: 78.5%; brownish red crystals; m.p.: 253–255°C; IR (KBr): 3446, 3141, 2518, 1710, 1637, 1616, 1511, 1483, 1461, 1324, 1214, 1091, 1054, 941, 796, 754, 597; ^{1}H-NMR (DMSO-d_6) δ: 1.24 (t, 3H, CH_3), 3.86 (q, 2H, N-CH_2), 6.87 (d, 1H, *J* = 7.24 Hz, 7′-H), 7.00 (dt, 1H, *J* = 8.01, 8.62 Hz, 5′-H), 7.10 (dd, 1H, *J* = 5.10, 7.87 Hz, 5-H), 7.39 (dt, 1H, *J* = 7.60, 8.79 Hz, 6′-H), 8.26 (dd, 1H, *J* = 1.58, 5.10 Hz, 6-H), 9.13 (d, 1H, *J* = 8.01 Hz, 4′-H), 9.34 (dd, 1H, *J* = 1.58, 7.87 Hz, 4-H), 10.99 (s, 1H, N′-H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 12.45 (CH_3), 33.53 (NCH_2), 109.58 ($C_{7'}$), 115.69 ($C_{5'}$), 117.65 (C_{3a}), 121.37 (C_5), 121.46 ($C_{3'a}$), 129.68 (C_3), 129.88 ($C_{4'}$), 133.38 ($C_{3'}$), 134.72 ($C_{6'}$), 136.37 (C_4), 144.75 ($C_{7'a}$), 149.42 (C_6), 156.89 (C_{1a}), 166.68 (C_2), 168.95 ($C_{2'}$); ESI-MS *m*/*z*: 292.0 [M^+ + H]. Anal. calcd. for $C_{17}H_{13}N_3O_2$ (291.3): C, 70.09; H, 4.50; N, 14.42. Found: C, 69.98; H, 4.36; N, 14.60.

(E)-3-(5-Chloro-2-oxoindolin-3-ylidene)-1-ethyl-1H-pyrrolo[2,3-b]pyridin-2(3H)-one ***11***

Yield: 73.8%; brownish red crystals; m.p.: 269–272°C; IR (KBr): 3415, 3195, 3133, 1695, 1618, 1589, 1454, 1359, 1315, 1259, 1103, 811, 790, 763, 611, 559; ^{1}H-NMR (DMSO-d_6) δ: 1.26 (t, 3H, CH_3), 3.86 (q, 2H, N-CH_2), 6.88 (d, 1H, *J* = 8.34 Hz, 7′-H), 7.11 (dd, 1H, *J* = 5.11, 7.90 Hz, 5-H), 7.45 (dd, 1H, *J* = 2.13, 8.34 Hz, 6′-H), 8.28 (dd, 1H, *J* = 1.58, 5.11 Hz, 6-H), 9.24 (d, 1H, *J* = 2.13 Hz, 4′-H), 9.34 (dd, 1H, *J* = 1.58, 7.90 Hz, 4-H), 11.16 (s, 1H, N′-H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 14.58 (CH_3), 35.47 (NCH_2), 110.88 ($C_{7'}$), 115.71 ($C_{5'}$), 118.13 (C_{3a}), 122.48 (C_5), 125.29 ($C_{3'a}$), 129.03 (C_3), 130.98 ($C_{4'}$), 132.43 ($C_{3'}$), 133.72 ($C_{6'}$), 136.65 (C_4), 143.37 ($C_{7'a}$), 149.98 (C_6), 156.92 (C_{1a}), 166.87 (C_2), 168.65 ($C_{2'}$); ESI-MS *m*/*z*: 326.1 [M^+ + H] (Cl = 35), 328.0 [M^+ + H] (Cl = 37). Anal. calcd. for $C_{17}H_{12}ClN_3O_2$ (325.06): C, 62.06; H, 3.71; N, 12.90. Found: C, 61.94; H, 3.87; N, 12.78.

(E)-1-Butyl-3-(5-fluoro-2-oxoindolin-3-ylidene)-1H-pyrrolo[2,3-b]pyridin-2(3H)-one ***12***

Yield: 74.9%; brownish red crystals; m.p.: 260–262°C; IR (KBr): 3421, 3303, 3130, 2956, 1704, 1589, 1465, 1359, 1257, 1199, 1101, 815, 788, 597; ^{1}H-NMR (DMSO-d_6) δ: 0.92 (t, 3H, CH_3), 1.35 (m, 2H, CH_2), 1.67 (m, 2H, CH_2), 3.82 (t, 2H, N-CH_2), 6.85 (d, 1H, *J* = 8.79 Hz, 7′-H), 7.11 (dd, 1H, *J* = 5.13, 7.89 Hz, 5-H), 7.26 (dd, 1H, *J* = 2.78, 8.79 Hz, 6′-H), 8.27 (dd, 1H, *J* = 1.59, 5.13 Hz, 6-H), 8.99 (d, 1H, *J* = 2.78 Hz, 4′-H), 9.35 (dd, 1H, *J* = 1.59, 7.89 Hz, 4-H), 11.01 (s, 1H, N′-H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 13.43 (CH_3), 19.49, 29.07 (CH_2), 38.31 (NCH_2), 110.24 ($C_{7'}$), 115.49 (C_{3a}), 116.21 (C_5), 118.05 ($C_{3'a}$), 119.50 (C_3), 121.92 ($C_{4'}$), 130.79 ($C_{3'}$), 134.50 ($C_{6'}$), 136.64 (C_4), 140.84 ($C_{7'a}$), 149.93 (C_6), 156.98 ($C_{5'}$), 158.00 (C_{1a}), 167.02 (C_2), 168.86 ($C_{2'}$); ESI-MS *m*/*z*: 338.1 [M^+ + H]. Anal. calcd. for $C_{19}H_{16}FN_3O_2$ (337.30): C, 67.65; H, 4.78; N, 12.46. Found: C, 67.22; H, 4.96; N, 12.44.

(E)-3-(5-Bromo-2-oxoindolin-3-ylidene)-1-butyl-1H-pyrrolo[2,3-b]pyridin-2(3H)-one ***13***

Yield: 81.4%; brownish red crystals; m.p.: 255–257°C; IR (KBr): 3421, 3301, 2956, 1702, 1618, 1589, 1454, 1359, 1309, 1257,

1101, 815, 594; ^{1}H-NMR (DMSO-d_6) δ: 0.92 (t, 3H, CH_3), 1.34 (m, 2H, CH_2), 1.67 (m, 2H, CH_2), 3.81 (t, 2H, N-CH_2), 6.83 (d, 1H, J = 8.33 Hz, 7′-H), 7.11 (dd, 1H, J = 5.10 Hz, 5-H), 7.56 (dd, 1H, J = 2.04, 8.33 Hz, 6′-H), 8.27 (dd, 1H, J = 1.58, 5.10 Hz, 6-H), 9.31 (d, 1H, J = 1.58 Hz, 4-H), 9.35 (d, 1H, J = 2.04 Hz, 4′-H), 11.15 (s, 1H, N′-H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 13.46 (CH_3), 19.52, 29.09 (CH_2), 40.00 (NCH_2), 111.49 ($C_{7'}$), 112.94 ($C_{5'}$), 115.47 (C_{3a}), 118.03 (C_5), 122.96 ($C_{3'a}$), 130.95 (C_3), 131.73 ($C_{4'}$), 133.66 ($C_{3'}$), 135.30 ($C_{6'}$), 136.66 (C_4), 143.68 ($C_{7'a}$), 149.96 (C_6), 156.93 (C_{1a}), 166.95 (C_2), 168.38 ($C_{2'}$); ESI-MS (m/z): 396.1 [M^- − H] (Br = 79), 398.1 [M^- − H] (Br = 81). Anal. calcd. for $C_{19}H_{16}BrN_3O_2$ (397.20): C, 57.30; H, 4.05; N, 10.55. Found: C, 57.22; H, 3.96; N, 10.44.

*(E)-1-Isopropyl-3-(2-oxoindolin-3-ylidene)-1H-pyrrolo[2,3-b]pyridin-2(3H)-one **14***

Yield: 72.8%; m.p.: 228–230°C; IR (KBr): 3432, 3151, 1700, 1618, 1438, 1400, 1384, 1336, 1103, 981; ^{1}H-NMR (DMSO-d_6) δ: 1.51 (d, 6H, 2 CH_3), 4.78 (m, 1H, N-CH), 6.86 (d, 1H, J = 7.55 Hz, 7′-H), 7.01 (t, 1H, J = 8.04, 15.50 Hz, 6′-H), 7.09 (dd, 1H, J = 5.09, 7.90 Hz, 5-H), 7.39 (dd, 1H, J = 7.67, 8.64 Hz, 5′-H), 8.25 (dd, 1H, J = 1.60, 5.09 Hz, 6-H), 9.10 (d, 1H, J = 7.67 Hz, 4′-H), 9.33 (dd, 1H, J = 1.60, 7.90 Hz, 4-H), 10.99 (s, 1H, N′-H); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 19.00 (2 CH_3), 43.10 (NCH), 109.74 ($C_{7'}$), 115.71 ($C_{5'}$), 117.61 (C_{3a}), 121.31 (C_5), 121.37 ($C_{3'a}$), 129.76 (C_3), 129.85 ($C_{4'}$), 133.40 ($C_{3'}$), 134.99 ($C_{6'}$), 136.26 (C_4), 144.71 ($C_{7'a}$), 149.31 (C_6), 156.72 (C_{1a}), 166.63 (C_2), 168.90 ($C_{2'}$); ESI-MS m/z: 306.1 [M^+ + H]. Anal. calcd. for $C_{18}H_{15}N_3O_2$ (305.3): C, 70.81; H, 4.95; N, 13.76. Found: C, 70.53; H, 5.04; N, 13.52.

Biological testing

MTT assay

The MTT cell proliferation assay [34] was used to test the antitumor activity of synthesized compounds. The cells were seeded in RPMI-1640 medium (100 μL) in a 96-well plate at a concentration of 5000 cells per well. After culturing for 12 h at 37°C and with 5% CO_2, cells were incubated with scalar concentrations of the tested compounds for 24 h. MTT was added to the cultures at a final concentration of 5 μg/mL, and incubated for 4 h. The formazane crystals were formed and dissolved in DMSO (100 μL) each well. The optical density was measured at 570 nm with the reference wavelength 630 nm. All of the compounds were tested thrice independently using the same DU145 cell line. IC_{50} (concentration that inhibits 50% of cell growth) was calculated using the Bacus Laboratories Incorporated Slide Scanner (Bliss) software, and the data in this manuscript represent mean of two independent experiments.

Inhibition on CDK2/cyclin A

Kinase inhibitor affinity measurement kit and related reagent were purchased from Invitrogen Corporation (Cat. No. PV3267, USA). Kinase activities were measured according to manufacturer instructions. Assays were typically performed in black, low-volume 384-well plates (Corning Part#3676). The data presented in this manuscript were generated using a Tecan Safire^2 plate reader (Tecan Group Ltd, Switzerland). Percent inhibition of tested compounds on CDK2/cyclin A was calculated by the following formula:

$$\text{Inhibition}\% = 100 \times (1 - T/C) \quad (1)$$

Where T is the percent phosphorylation of the tested compounds, and C is the percent phosphorylation of no inhibition (control).

Determination of the expression of CDK2/Cyclin D1 and p27 by Western blot

Androgen-independent prostate carcinoma cell line DU145 cells were cultured in six-well plates in 10% FBS medium and treated with different concentrations of compounds **7** and **9** for 24 h. Cellular proteins were extracted in lysis buffer containing 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 μg/mL PMSF, 30 μg/mL aprotinin, and 50 Mm Tris-HCl (pH = 8.0). The samples were then placed on ice for 10 min and centrifuged at 14 000 rpm for 30 min at 4°C. Protein concentrations were measured using a BCA protein assay kit (Pierce chemical, Rockford, IL, USA). 100 μg of protein extracts were subjected to SDS-PAGE using 8–15% (depending on the protein size to be analyzed) and electro-transferred to a Hybond-C nitrocellulose membrane at 150 V for 1 h at 4°C. The nitrocellulose membrane was incubated overnight in a blocking solution containing 15% bovine skim milk in 10 mM PBS, followed by incubating for 1 h with antiphospho-CDK2^{Thr160}, anti-p27, anti-Cyclin-D1 (all from Santa Cruz, Inc., Santa Cruz, CA, USA), or anti-β-actin (Sigma, USA) antibody, respectively, and then with a second antibody. After three washes with PBS containing 0.1% Tween 20, incubated in ECL solution (NENTM Life Science Inc., Boston, MA, USA) for 2 min, the protein signals in the membrane was detected by an X-ray film.

The authors have declared no conflict of interest.

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