Ebrahim Saeedian Moghadam, Farhad Saravani, Seyednasser Ostad, Shohreh Tavajohi, Morteza Pirali Hamedani and Mohsen Amini*

Design, synthesis and cytotoxicity evaluation of indibulin analogs

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Abstract: Indibulin is one of the most potent tubulin polymerization inhibitors with minimal peripheral neuropathy. The design and synthesis of new indibulin analogs were carried out in order to investigate their anti-cancer activity. The target compounds **4a–i** were synthesized in multistep reactions starting with the related indole derivatives. Compound **4f** shows the highest cytotoxic activity on HT-29 and Caco-2 cell lines with the respective half maximal inhibitory concentration (IC_{50}) values of 5.1 μM and 7.3 μM. In the case of the T47-D cell line, compound **4c** exerts the best cytotoxic activity with an IC_{50} value of 11.5 μM. In the cell cycle analysis on HT-29 cells, compound **4f** at 5.1 μM showed an increase in the percentage of cells in the sub-G1 phase. Altogether, nine target compounds were synthesized and characterized by infrared spectroscopy (IR), proton nuclear magnetic resonance (^{1}H NMR), carbon-13 nuclear magnetic resonance (^{13}C NMR), mass spectrometry (MS) and elemental analysis. Some of the compounds show good cytotoxic activity against cancerous cell lines.

Keywords: anti-cancer; indibulin analogs; MTT; pyrrole.

Dedicated to: This manuscript is dedicated to the fond memory of Professor Abbas Shafiee.

Introduction

Cancer is a complex group of genetic disorders involving an abnormal and uncontrolled growth of cells with the ability to spread throughout the body [1, 2]. Despite tireless efforts over the decades for prevention, diagnosis and therapy, cancer remains a major leading cause of mortality worldwide. It is the second cause of death in North America and Europe after cardiovascular diseases [3–6]. Chemotherapy is one of the most effective techniques used for cancer treatment. Unfortunately, the lack of selectivity and emerging drug resistance decrease the efficacy and potential of cancer chemotherapy, and further efforts for finding new anti-cancer drugs are necessary [7–9].

As a part of eukaryote cells, microtubules play an important position in cell biology. Microtubules play a role in adjusting and tuning of the various basic cellular functions including organelle transport, mitosis processes, adjustment of motility, formation and in keeping cell morphology and cell signaling intact [10]. During the last few years, laboratory research and clinical investigation on anti-tubulin agents for applying these compounds in the treatment of a large number of cancers have rapidly increased. The rate of tubulin polymerization affects the induction of apoptosis in cells. The main family of tubulin polymerization inducer is taxane and its derivatives. Paclitaxel and docetaxel exert their anti-tubulin effect by binding to the taxol binding site of tubulin and by doing so, they increase the rate of tubulin polymerization. In addition to the taxane family, there is a large number of compounds including combretastatin A4, indibulin and nocodazole which attach to the colchicine binding site of tubulin and cause a decrease in the tubulin polymerization rate. With respect to the high level of cell proliferation in cancerous cells and the effect of tubulin disrupting agents on the inhibition of the mitosis process, these compounds could control cell division in cancerous cells [11].

Anti-mitotic agents such as paclitaxel and some of the compounds that target the colchicine binding site lead to the emergence of peripheral neuropathy as an unfortunate side effect. Indibulin with a high level of anti-cancer activity by anti-mitotic mechanism does not exert peripheral neuropathy, and this feature distinguishes indibulin from other similar acting compounds. It seems that indibulin has the ability to discriminate between mature neuronal and non-neuronal tubulin [12]. Efforts are being continued to find new tubulin polymerization inhibitors inspired by the structure of indibulin [13–15] (Figure 1A). For example,

***Corresponding author: Mohsen Amini,** Department of Medicinal Chemistry, Faculty of Pharmacy and Drug Design and Development Research Center, The Institute of Pharmaceutical Sciences (TIPS), Tehran University of Medical Sciences, Tehran 1417614411, Iran, e-mail: moamini@tums.ac.ir
Ebrahim Saeedian Moghadam, Farhad Saravani and Morteza Pirali Hamedani: Department of Medicinal Chemistry, Faculty of Pharmacy and Drug Design and Development Research Center, The Institute of Pharmaceutical Sciences (TIPS), Tehran University of Medical Sciences, Tehran 1417614411, Iran
Seyednasser Ostad and Shohreh Tavajohi: Department of Toxicology and Pharmacology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran 1417614411, Iran

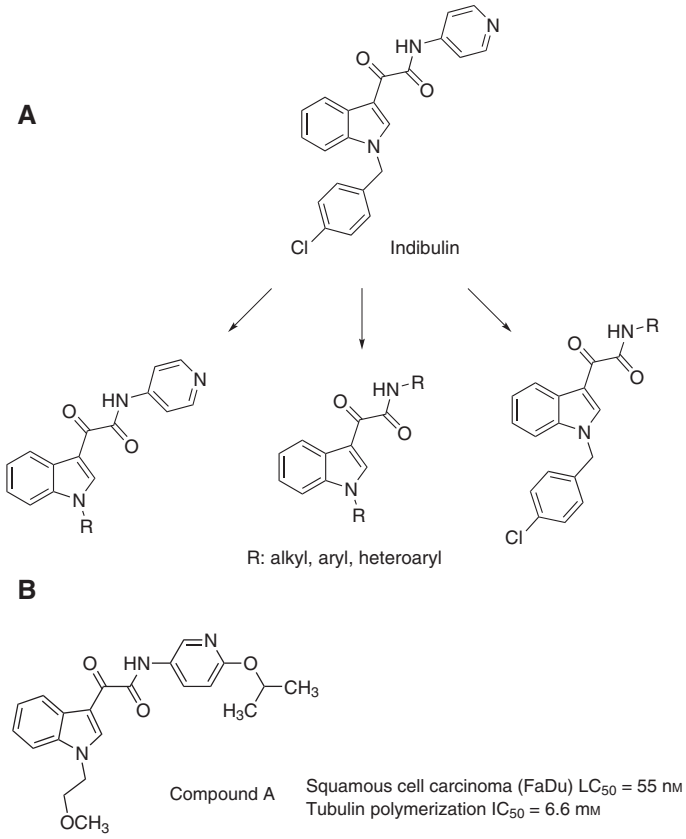


Figure 1 Previous structural modifications of indibulin (A), compound A with significant head and neck tumor growth inhibition (B).

a remarkable tumor growth inhibition in a mouse xenograft model of head and neck cancer has been observed in the case of indibulin-related compound A (Figure 1B) [13]. In addition to these derivatives, a large number of other pyrrole-based bioactive compounds have been synthesized [16–19].

In continuation of our previous efforts [20–25] in the design and synthesis of new chemotherapeutic agents, in this paper we report the synthesis of a series of indibulin-related *N*-benzylpyrrole derivatives and the evaluation of their anti-cancer activity on three diverse cancer cell lines including human breast cancer tumor (T47-D), human colon adenocarcinoma (Caco-2 and HT-29) using mouse fibroblast normal cells (NIH-3T3) as a control. The structural modifications are explained in Figure 2.

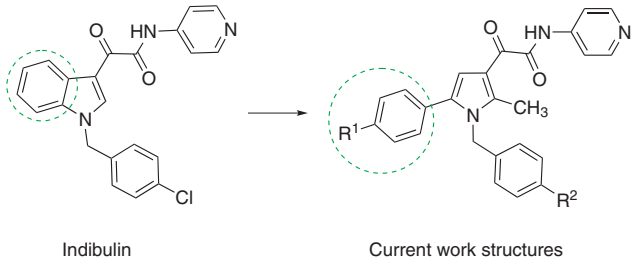
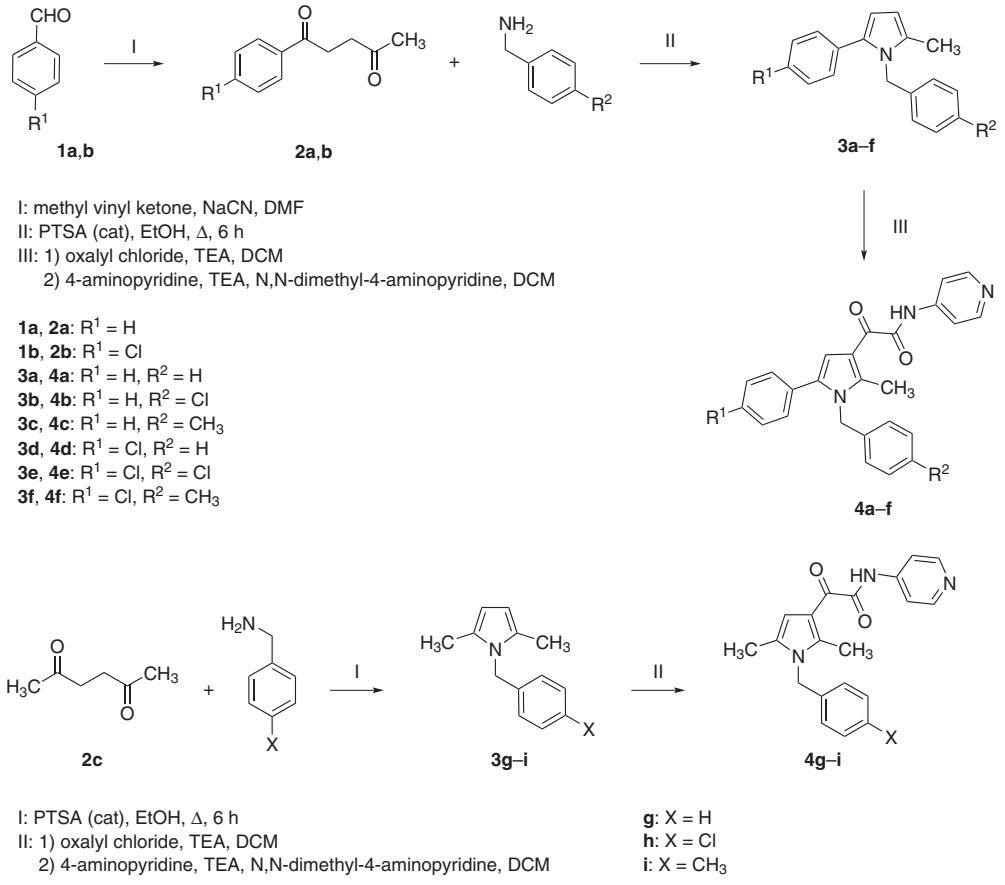


Figure 2 Structural changes on indibulin in the current work.

Results and discussion

The synthetic pathway to final compounds is shown in Scheme 1. In the first step, the Stetter reaction of benzaldehyde derivatives **1a–b** with methyl vinyl ketone in the presence of a catalytic amount of sodium cyanide in dimethylformamide (DMF) yielded 1-aryl-1,4-pentanediones **2a–b**. Then, under the Paal-Knorr conditions, diones **2a–c** were allowed to react with appropriate benzylamine derivatives in the presence of *p*-toluenesulfonic acid (PTSA) as a catalyst in refluxing ethanol for 6 h to obtain the corresponding *N*-benzylpyrrole derivatives **3a–i**. These pyrroles were converted into final target compounds **4a–i** by treatment with oxalyl chloride in dichloromethane in the presence of triethylamine at room temperature, followed by the reaction of the intermediate acid chloride with 4-aminopyridine.

Compounds **4a–i** were investigated for their cytotoxicity against three cancer cell lines HT-29, Caco-2 and T47-D



I: methyl vinyl ketone, NaCN, DMF
II: PTSA (cat), EtOH, Δ, 6 h
III: 1) oxalyl chloride, TEA, DCM
2) 4-aminopyridine, TEA, N,N-dimethyl-4-aminopyridine, DCM

1a, **2a**: R^1 = H
1b, **2b**: R^1 = Cl
3a, **4a**: R^1 = H, R^2 = H
3b, **4b**: R^1 = H, R^2 = Cl
3c, **4c**: R^1 = H, R^2 = CH_3
3d, **4d**: R^1 = Cl, R^2 = H
3e, **4e**: R^1 = Cl, R^2 = Cl
3f, **4f**: R^1 = Cl, R^2 = CH_3

I: PTSA (cat), EtOH, Δ, 6 h
II: 1) oxalyl chloride, TEA, DCM
2) 4-aminopyridine, TEA, N,N-dimethyl-4-aminopyridine, DCM

g: X = H
h: X = Cl
i: X = CH_3

Scheme 1 Synthetic pathway to compounds **4a–i.**

Table 1 *In vitro* cytotoxic activities (IC_{50}, μM) of compounds **4a–i**.

Compound	NIH-3T3	HT-29	Caco-2	T47-D
4a	32.1±1.9	25.3±4.1	49.1±1.4	41.1±2.1
4b	17±2.2	27.5±1.5	8.3±1.9	>100
4c	16.5±2.7	31.4±3.3	9.8±1.3	11.5±2.9
4d	12±1.1	32.2±3.5	36.3±2.1	>100
4e	25.6±3.0	21.2±2.7	29.5±2.7	20.5±1.3
4f	17.4±1.7	5.1±1.8	7.3±1.8	26.4±1.8
4g	50.6±2.6	7.7±2.4	75.1±2.0	40.4±2.0
4h	30.4±3.5	28.7±1.6	58.2±1.5	46.3±2.2
4i	65.8±2.9	58.6±3.7	82.8±2.3	61.1±4.1
Paclitaxel	8.7±3.1	19.4±1.9	17.3±2.4	0.35±0.2

with a normal cell line NIH-3T3 as control (Table 1). The screening started with the HT-29 cell line with the tested compounds showing cytotoxicity with half maximal inhibitory concentration (IC_{50}) values between 5.1 and 58.6 μM. The most active against HT-29 are compounds **4f** and **4g**. With the Caco-2 cell line, the most active compounds **4f**, **4b** and **4c** show anti-cancer activity with an IC_{50} of less than 10 μM (7.3, 8.3 and 9.8 μM, respectively). In the case of T47-D cell line, compound **4c** is the most active with an IC_{50} value of 11.5 μM. Interestingly, compounds **4f** and **4g** against HT-29 and compounds **4f**, **4b**, **4c** against Caco-2 are more active than paclitaxel, the reference drug. In addition, all the tested compounds have lower toxicity on NIH-3T3 normal cell line than the standard drug.

With regard to the cytotoxic activity of **4f**, we further evaluated its effect on cell cycle distribution of cultured HT-29 cells, as determined by flow cytometry (not shown). After treatment of cells with **4f** at IC_{50} concentration for 24 h, this compound caused an increase in the percentage of cells in the sub-G1 phase, representing an increase in cell death which could be consistent with the induction of apoptosis.

Conclusions

A series of indibulin-related *N*-benzylpyrrole derivatives were synthesized and investigated for their cytotoxic activity against four diverse cell lines. Compounds **4f** and **4g** show the highest cytotoxic activity against the HT-29 cell line. Cell cycle analysis shows arrest in the sub-G1 phase of cells treated with **4f** which could lead to apoptosis induction and cell death.

Experimental

Melting points were taken on a Kofler hot-stage apparatus (Reichert, Vienna, Austria) and are uncorrected. The proton nuclear magnetic resonance (^{1}H NMR) spectra (500 MHz) and carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra (125 MHz) were recorded on a Bruker FT-500 MHz spectrometer using $CDCl_3$ as the solvent. Elemental analyses were carried out using a Perkin Elmer Model 240-C apparatus. Infrared (IR) spectra were recorded using KBr pellets on a Nicolet Magna 550-FT spectrometer. Mass spectra were recorded on an Agilent 5975C spectrometer. 1-Phenylpentane-1,4-dione (**2a**) and 1-(4-chlorophenylpentane)-1,4-dione (**2b**) were prepared using the Stetter reaction as previously reported [26, 27]. Compound **2c** was commercially available.

General procedure for the preparation of *N*-benzylpyrrole derivatives 3a–i

A mixture of appropriate diketone **2a–c** (1 mmol), a benzylamine derivative (1.1 mmol) and a catalytic amount of PTSA in ethanol was heated under reflux for 6 h. After the completion of the reaction, the mixture was concentrated and the residue of **3a–i** was purified by flash chromatography (hexane/ethyl acetate 10:1 as an eluent) [28]. Compounds **3a**, **3d**, **3g**, **3h** and **3i** have been reported previously [29–32].

1-(4-Chlorobenzyl)-2-methyl-5-phenyl-1*H*-pyrrole (3b) Light yellow solid; yield 51%; mp 83–84°C; IR: 3063, 3027, 2972, 2935, 1599, 1513, 1489, 1447, 1400, 1312, 1088, 1011, 816, 750, 699 cm^{-1}; ^{1}H NMR: δ 2.13 (s, 3H, C$\underline{H}_3$), 5.08 (s, 2H, C$\underline{H}_2$), 6.04 (s, 1H, pyrrole), 6.21 (s, 1H, pyrrole), 6.83 (d, J = 7.5 Hz, 2H, Ar), 7.21–7.29 (m, 7H, Ar); ^{13}C NMR: 12.6, 47.0, 107.5, 108.3, 126.8, 127.0, 128.5, 128.6, 128.9, 130.2, 132.8, 133.7, 134.6, 137.5; MS: *m/z* 281 (84), 156 (100), 125 (84), 89 (16%). Anal. Calcd for $C_{18}H_{16}ClN$: C, 76.72; H, 5.72; N, 4.97. Found: C, 76.61; H, 5.66; N, 5.03.

2-Methyl-1-(4-methylbenzyl)-5-phenyl-1*H*-pyrrole (3c) Light brown solid; yield 57%; mp 61–62°C; IR: 3054, 3024, 2922, 2860, 1601, 1513, 1444, 1400, 1353, 1311, 1025, 799, 751, 700 cm^{-1}; ^{1}H NMR: δ 2.09 (s, 3H, CH_3), 2.26 (s, 3H, CH_3), 5.04 (s, 2H, CH_2), 6.02 (s, 1H, pyrrole), 6.21 (s, 1H, pyrrole), 6.79 (d, J = 7.5 Hz, 2H, Ar), 7.04 (d, J = 7.5 Hz, 2H, Ar), 7.14 (t, J = 7.0 Hz, 1H, Ar), 7.21 (t, J = 7.0 Hz, 2H, Ar), 7.27 (d, J = 7.0 Hz, 2H, Ar); ^{13}C NMR: 12.6, 21.1, 47.5, 107.2, 108.0, 125.6, 126.6, 128.4, 128.6, 129.4, 130.4, 133.9, 134.6, 136.0, 136.5; MS: *m/z* 261 (40), 156 (30), 125 (17), 105 (100), 77 (13%). Anal. Calcd for $C_{19}H_{19}N$: C, 87.31; H, 7.33; N, 5.36. Found: C, 87.39; H, 7.27; N, 5.34.

1-(4-Chlorobenzyl)-2-(4-chlorophenyl)-5-methyl-1*H*-pyrrole (3e) Light brown oil; yield 50%; IR: 3053, 2927, 2854, 1511, 1492, 1466, 1265, 1095, 1014, 832, 739, 706 cm^{-1}; ^{1}H NMR: δ 2.09 (s, 3H, CH_3), 5.02 (s, 2H, CH_2), 6.03 (s, 1H, pyrrole), 6.19 (s, 1H, pyrrole), 6.79 (d, J = 8.0 Hz, 2H, Ar), 7.14 (d, J = 8.0 Hz, 2H, Ar), 7.20–7.25 (m, 4H, Ar); ^{13}C NMR: 12.4, 46.9, 107.7, 108.6, 126.8, 128.5, 128.9, 129.6, 130.5, 132.0, 132.6, 132.9, 133.1, 137.2; MS: *m/z* 315 (10), 190 (16), 125 (40), 100 (67), 57 (100%). Anal. Calcd for $C_{18}H_{15}Cl_2N$: C, 68.37; H, 4.78; N, 4.43. Found: C, 68.28; H, 4.73; N, 4.46.

2-(4-Chlorophenyl)-5-methyl-1-(4-methylbenzyl)-1*H*-pyrrole (3f) Light brown oil; yield 59%; IR: 3048, 2926, 2858, 1517, 1463, 1385, 1263, 1112, 912, 839, 737, 701 cm^{-1}; ^{1}H NMR: δ 2.12 (s, 3H, CH_3), 2.30 (s, 3H, CH_3), 5.04 (s, 2H, CH_2), 6.02 (s, 1H, pyrrole), 6.19 (s, 1H, pyrrole), 6.78 (d, J = 7.5 Hz, 2H, Ar), 7.08 (d, J = 7.5 Hz, 2H, Ar), 7.18 (d, J = 7.5 Hz, 2H, Ar), 7.21 (d, J = 7.5 Hz, 2H, Ar); ^{13}C NMR: δ 12.4, 20.9, 47.2, 107.2, 108.2, 125.3, 128.4, 129.4, 129.5, 130.7, 132.2, 132.3, 133.1, 135.6, 136.5; MS: *m/z* 295 (15), 197 (11), 149 (14), 111 (30), 97 (52), 83 (54), 57 (100%). Anal. Calcd for $C_{19}H_{18}ClN$: C, 77.15; H, 6.13; N, 4.74. Found: C, 77.06; H, 6.17; N, 4.72.

General procedure for the preparation of substituted acetamides 4a–i

A solution of pyrrole **3a–i** (1 mmol) and triethylamine (1.2 mmol) in dichloromethane (10 mL) was treated dropwise at 0°C with a solution of oxalyl chloride (1.1 mmol) in dichloromethane (5 mL). The mixture was stirred at room temperature for 4 h and then concentrated [33]. The residue was dissolved in dichloromethane and the solution was treated with triethylamine (1.2 mmol), 4-aminopyridine and a catalytic amount of *N,N*-dimethyl-4-aminopyridine. The mixture was stirred at room temperature for 12 h, concentrated and the residue was subjected to silica gel column chromatography eluting with hexane/ethyl acetate (3:1) to obtain the analytically pure product **4a–i**. Compounds **4b** and **4h** have been mentioned previously in the literature but not fully characterized [34].

2-(1-Benzyl-2-methyl-5-phenyl-1*H*-pyrrol-3-yl)-2-oxo-*N*-(pyridin-4-yl)acetamide (4a) Yellow solid; yield 41%; mp 161–163°C; IR: 3280, 2925, 2858, 1697, 1641, 1581, 1502, 1475, 1135, 995, 833, 757, 698 cm^{-1}; ^{1}H NMR: δ 2.54 (s, 3H, CH_3), 5.18 (s, 2H, CH_2), 6.93 (d, J = 7.0 Hz, 2H, Ar), 7.24–7.34 (m, 8H, Ar), 7.49 (s, 1H, pyrrole), 7.79 (d, J = 5.0 Hz, 2H, pyridine), 8.57 (d, J = 5.0 Hz, 2H, pyridine), 9.65 (s, 1H, NH); ^{13}C NMR: δ 13.0, 47.8, 112.4, 113.9, 116.3, 125.4, 127.6, 128.1, 128.5, 129.0, 129.1, 131.5, 135.6, 136.4, 143.4, 145.8, 148.5, 161.2, 179.7; MS: *m/z* 395 (21), 274 (100), 91 (72), 57 (10%). Anal. Calcd for $C_{25}H_{21}N_3O_2$: C, 75.93; H, 5.35; N, 10.63. Found: C, 75.80; H, 5.32; N, 10.69.

2-(1-(4-Chlorobenzyl)-2-methyl-5-phenyl-1*H*-pyrrol-3-yl)-2-oxo-*N*-(pyridin-4-yl)acetamide (4b) Yellow solid; yield 39%; mp 195–197°C; IR: 3335, 2923, 2852, 1708, 1632, 1593, 1506, 1473, 1350, 813, 763, 698, 556 cm^{-1}; ^{1}H NMR: δ 2.54 (s, 3H, C$\underline{H}_3$), 5.14 (s, 2H, C$\underline{H}_2$), 6.85 (d, J = 8.0 Hz, 2H, Ar), 7.25–7.38 (m, 7H, Ar), 7.52 (s, 1H, pyrrole), 7.63 (d, J = 5.5 Hz, 2H, pyridine), 8.56 (d, J = 5.5 Hz, 2H, pyridine), 9.40 (s, 1H, NH); ^{13}C NMR: δ 12.8, 47.2, 112.7, 113.6, 116.6, 126.9, 128.1, 128.6, 129.1, 129.2, 131.5, 133.5, 135.1, 135.3, 142.7, 143.9, 150.7, 150.9, 161.0, 180.4; MS: *m/z* 429 (11), 308 (54), 236 (11), 125 (59), 103 (53), 73 (100), 51 (7%). Anal. Calcd for $C_{25}H_{20}ClN_3O_2$: C, 69.85; H, 4.69; N, 9.77. Found: C, 69.89; H, 4.60; N, 9.74.

2-(2-Methyl-1-(4-methylbenzyl)-5-phenyl-1*H*-pyrrol-3-yl)-2-oxo-*N*-(pyridin-4-yl)acetamide (4c) Yellow solid; yield 34%; mp 145–147°C; IR: 3279, 2920, 1704, 1636, 1605, 1512, 1416, 1354, 1135, 828, 804, 695, 569 cm^{-1}; ^{1}H NMR: δ 2.33 (s, 3H, CH_3), 2.54 (s, 3H, CH_3), 5.14 (s, 2H, CH_2), 6.82 (d, J = 8.0 Hz, 2H, Ar), 7.12 (d, J = 8.0 Hz, 2H, Ar), 7.27–7.36 (m, 5H, Ar), 7.51 (s, 1H, pyrrole), 7.69 (d, J = 5.5 Hz, 2H, pyridine), 8.56 (d, J = 5.5 Hz, 2H, pyridine), 9.50 (s, 1H, NH); ^{13}C NMR: δ

13.0, 21.0, 47.6, 112.5, 113.7, 116.4, 125.4, 128.0, 128.5, 129.2, 129.6, 131.7, 133.5, 135.6, 137.3, 143.3, 144.7, 149.8, 149.9, 161.2, 180.1; MS: *m/z* 409 (25), 308 (20), 288 (90), 183 (8), 125 (18), 105 (100%). Anal. Calcd for $C_{26}H_{23}N_3O_2$: C, 76.26; H, 5.66; N, 10.26. Found: C, 76.20; H, 5.61; N, 10.27.

2-(1-Benzyl-5-(4-chlorophenyl)-2-methyl-1*H*-pyrrol-3-yl)-2-oxo-*N*-(pyridin-4-yl) acetamide (4d) Yellow solid; yield 37%; mp 132–134°C; IR: 3353, 2923, 2846, 1703, 1634, 1589, 1503, 1416, 1136, 824, 724, 661 cm^{-1}; ^1H NMR: δ 2.55 (s, 3H, CH_3), 5.14 (s, 2H, CH_2), 6.91 (d, J = 7.0 Hz, 2H, Ar), 7.21 (d, J = 7.5 Hz, 2H, Ar), 7.29 (d, J = 7.5 Hz, 2H, Ar), 7.30–7.35 (m, 3H, Ar), 7.52 (s, 1H, pyrrole), 7.63 (d, J = 5.5 Hz, 2H, pyridine), 8.56 (d, J = 5.5 Hz, 2H, pyridine), 9.43 (s, 1H, NH); ^{13}C NMR: δ 12.8, 47.7, 113.0, 113.6, 116.5, 125.4, 127.7, 128.7, 129.0, 130.1, 130.4, 131.4, 134.1, 136.3, 143.2, 143.9, 150.7, 150.9, 161.1, 180.3; MS: *m/z* 429 (17), 308 (100), 125 (88), 89 (11%). Anal. Calcd for $C_{25}H_{20}ClN_3O_2$: C, 69.85; H, 4.69; N, 9.77. Found: C, 69.74; H, 4.65; N, 9.79.

2-(1-(4-Chlorobenzyl)-5-(4-chlorophenyl)-2-methyl-1*H*-pyrrol-3-yl)-2-oxo-*N*-(pyridin-4-yl) acetamide (4e) Yellow solid; yield 38%; mp 157–159°C; IR: 3343, 2928, 1693, 1649, 1590, 1500, 1406, 1137, 1091, 809, 750, 557 cm^{-1}; ^1H NMR: δ 2.53 (s, 3H, CH_3), 5.10 (s, 2H, CH_2), 6.83 (d, J = 8.4 Hz, 2H, Ar), 7.18 (d, J = 8.4 Hz, 2H, Ar), 7.26–7.32 (m, 4H, Ar), 7.49 (s, 1H, pyrrole), 7.70 (d, J = 6.1 Hz, 2H, pyridine), 8.56 (d, J = 6.1 Hz, 2H, pyridine), 9.51 (s, 1H, NH); ^{13}C NMR: δ 12.8, 47.1, 113.0, 113.7, 116.5, 126.7, 128.8, 129.3, 129.9, 130.3, 133.6, 134.0, 134.3, 134.7, 143.0, 144.7, 149.8, 161.0, 180.1; MS: *m/z* 463 (13), 435 (5), 342 (77), 125 (100), 89 (11%). Anal. Calcd for $C_{25}H_{19}Cl_2N_3O_2$: C, 64.67; H, 4.12; N, 9.05. Found: C, 64.57; H, 4.10; N, 9.03.

2-(5-(4-Chlorophenyl)-2-methyl-1-(4-methylbenzyl)-1*H*-pyrrol-3-yl)-2-oxo-*N*-(pyridin-4-yl) acetamide (4f) Yellow solid; yield 33%; mp 150–151°C; IR: 3353, 2920, 2849, 1700, 1638, 1593, 1505, 1477, 1129, 827, 805, 751, 658 cm^{-1}; ^1H NMR: δ 2.33 (s, 3H, CH_3), 2.55 (s, 3H, CH_3), 5.11 (s, 2H, CH_2), 6.80 (d, J = 7.5 Hz, 2H, Ar), 7.13 (d, J = 7.5 Hz, 2H, Ar), 7.22 (d, J = 8.0 Hz, 2H, Ar), 7.29 (d, J = 8.0 Hz, 2H, Ar), 7.52 (s, 1H, pyrrole), 7.63 (d, J = 5.2 Hz, 2H, pyridine), 8.56 (d, J = 5.2 Hz, 2H, pyridine), 9.41 (s, 1H, NH); ^{13}C NMR: δ 12.8, 21.0, 47.6, 112.9, 113.6, 116.4, 125.3, 128.7, 129.7, 130.2, 130.3, 133.2, 134.0, 134.1, 137.4, 143.3, 143.9, 150.7, 150.9, 161.1, 180.2; MS: *m/z* 443 (28), 322 (90), 218 (7), 105 (100), 79 (18%). Anal. Calcd for $C_{26}H_{22}ClN_3O_2$: C, 70.35; H, 5.00; N, 9.47. Found: C, 70.46; H, 5.03; N, 9.49.

2-(1-Benzyl-2,5-dimethyl-1*H*-pyrrol-3-yl)-2-oxo-*N*-(pyridin-4-yl) acetamide (4g) Yellow solid; yield 31%; mp 150–152°C; IR: 3365, 2923, 2851, 1703, 1634, 1577, 1499, 1412, 1356, 1137, 823, 752, 660 cm^{-1}; ^1H NMR: δ 2.16 (s, 3H, CH_3), 2.55 (s, 3H, CH_3), 5.09 (s, 2H, CH_2), 6.92 (d, J = 7.5 Hz, 2H, Ar), 7.19 (s, 1H, pyrrole), 7.24–7.34 (m, 3H, Ar), 7.63 (d, J = 5.5 Hz, 2H, pyridine), 8.55 (d, J = 5.5 Hz, 2H, pyridine), 9.43 (s, 1H, NH); ^{13}C NMR: δ 12.1, 12.6, 46.9, 110.7, 113.6, 115.6, 125.4, 127.7, 129.5, 129.6, 135.9, 142.0, 144.1, 150.6, 150.8, 161.4, 179.9; MS: *m/z* 333 (12), 212 (100), 184 (4), 121 (6), 91 (80%). Anal. Calcd for $C_{20}H_{19}N_3O_2$: C, 72.05; H, 5.74; N, 12.60. Found: C, 72.12; H, 5.80; N, 12.61.

2-(1-(4-Chlorobenzyl)-2,5-dimethyl-1*H*-pyrrol-3-yl)-2-oxo-*N*-(pyridin-4-yl)acetamide (4h) Yellow solid; yield 27%; mp 129–131°C; IR: 3350, 2921, 2852, 1707, 1594, 1479, 1416, 1131, 879, 824, 754, 553 cm^{-1}; ^1H NMR: δ 2.15 (s, 3H, CH_3), 2.53 (s, 3H, CH_3), 5.06 (s, 2H, CH_2), 6.85 (d, J = 8.1 Hz, 2H, Ar), 7.19 (s, 1H, pyrrole), 7.30 (d, J = 8.1 Hz, 2H, Ar), 7.63 (d, J = 6.2 Hz, 2H, pyridine), 8.56 (d, J = 6.2 Hz, 2H, pyridine), 9.41 (s, 1H, NH); ^{13}C NMR: δ 12.1, 12.5, 46.3, 110.9, 113.5, 115.7, 126.9, 129.2, 129.3, 133.6, 134.4, 141.7, 144.0, 150.7, 150.8, 161.3, 180.0; MS: *m/z* 367 (8), 342 (28), 246 (67), 125 (100), 89 (11), 57 (10%). Anal. Calcd for $C_{20}H_{18}ClN_3O_2$: C, 65.31; H, 4.93; N, 11.42. Found: C, 65.22; H, 4.90; N, 11.36.

2-(2,5-Dimethyl-1-(4-methylbenzyl)-1*H*-pyrrol-3-yl)-2-oxo-*N*-(pyridin-4-yl)acetamide (4i) Yellow solid; yield 35%; mp 142–143°C; IR: 3340, 2922, 2855, 1708, 1618, 1594, 1502, 1476, 1411, 1036, 824, 753, 550 cm^{-1}; ^1H NMR: δ 2.17 (s, 3H, CH_3), 2.33 (s, 3H, CH_3), 2.55 (s, 3H, CH_3), 5.06 (s, 2H, CH_2), 6.81 (d, J = 7.5 Hz, 2H, Ar), 7.13 (d, J = 7.5 Hz, 2H, Ar), 7.18 (s, 1H, pyrrole), 7.66 (d, J = 5.0 Hz, 2H, pyridine), 8.56 (d, J = 5.0 Hz, 2H, pyridine), 9.47 (s, 1H, NH); ^{13}C NMR: δ 12.1, 12.6, 21.0, 46.7, 110.5, 113.6, 115.4, 125.4, 129.5, 129.6, 132.8, 137.4, 142.2, 144.4, 150.2, 161.4, 179.6; MS: *m/z* 347 (7), 322 (16), 226 (52), 121 (6), 105 (100), 79 (10%). Anal. Calcd for $C_{21}H_{21}N_3O_2$: C, 72.60; H, 6.09; N, 12.10. Found: C, 72.69; H, 6.14; N, 12.09.

Cell lines

Four cell lines, namely human breast adenocarcinoma (T47-D), human colon adenocarcinoma (Caco-2 and HT-29) and mouse embryo fibroblast (NIH-3T3), were obtained from the Pasteur Institute (Tehran, Iran). The cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Roche, Germany) at 37°C in a humidified incubator with 5% CO_2. In the case of the Caco-2 cell line, the final cell culture medium contained RPMI-1640 (55%) and Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) (35%) plus the supplements mentioned above.

Cytotoxicity evaluation by the MTT assay

All the final compounds were investigated for their antiproliferative potential at 0.01–100 μM concentrations. Two controls were prepared within each 96-well plate: a solvent [dimethyl sulfoxide (DMSO)] control and a positive control (paclitaxel). Briefly, appropriate cells were seeded in 96-well plates (Nunc, Roskilde, Denmark) at a density of 10 000 viable cells per well and incubated for 24 h to allow for cell attachment. Solutions of the compounds were prepared by serial dilution from the stock solution and added to each well. Cells were then incubated with the compounds for another 48 h. The response of cells to compounds was evaluated by determining the cell survival using 3-(4,5-dimethylthiazoyl-2-yl) 2,5-diphenyl tetrazolium bromide (MTT, Carl Roth, Karlsruhe, Germany). At first, cells were washed with phosphate buffered saline (PBS) and then 20 μL of the MTT reagent (5 mg/mL) in PBS was added to each well. After 4 h of incubation at 37°C, the medium was discarded, DMSO (100 μL) was added to each well and the plates were vigorously shaken to dissolve the purple tetrazolium crystals in DMSO. The absorbance of each well was measured using a plate reader (Anthous 2020; Austria) at a test wavelength of 550 nm against a standard reference solution at 690 nm. Assays were performed in triplicate in three independent experiments, and the concentration required for 50% inhibition of cell viability (IC_{50}) was calculated by plotting the percentage cytotoxicity versus the concentration on a logarithmic graph [35].

Flow cytometric analysis of cell cycle distribution

For flow cytometric analysis of DNA content, 1×10^6 HT-29 cells were treated with compound **4f** (at IC_{50} concentration) for 24 h. After centrifugation, for cell fixation, 0.7 mL of cold 75% ethanol was added to samples on ice for at least 3 h. Cells were washed with PBS and resuspended in 0.25 mL of PBS, with 5 μL of 10 mg/mL RNase A and Triton X-100 (0.1%). Cells were incubated at 37°C for 1 h and then treated with 10 μL of 50 μg/mL propidium iodide (PI). Fluorescence was measured using an FACSCalibur flow cytometer (BDBiosciences, San Jose, CA, USA) [35].

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