Synthesis and anti-breast cancer activity of novel indibulin related diarylpyrrole derivatives

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Abstract

Background During recent years, a number of anti-tubulin agents were introduced for treatment of diverse types of cancer. Despite their potential in the treatment of cancer, drug resistance and adverse toxicity, such as peripheral neuropathy, are some of the negative effects of anti-tubulin agents. Among anti-tubulin agents, indibulin was found to cause minimal peripheral neuropathy. Thus far, however, indibulin has not entered clinical usage, caused in part by its poor aqueous solubility and other developmental problems in preclinical evaluation.

Objectives With respect to need for finding potent and safe anticancer agents, in our current research work, we synthesized several indibulin-related diarylpyrrole derivatives and investigated their anti-cancer activity.

Methods Cell culture studies were performed using the MTT cell viability assay on the breast cancer cell lines MCF-7, T47-D, and MDA-MB231 and also NIH-3 T3 cells as representative of a normal cell line. The activity of some of the synthesized compounds for tubulin interaction was studied using colchicine binding and tubulin polymerization assays. The annexin V-FITC/PI method and flow cytometric analysis were used for studying apoptosis induction and cell cycle distribution.

Results and conclusion Two of the synthesized compounds, **4f** and **4g**, showed high activity on the MDA-MB231 cell line (IC_{50} = 11.82 and 13.33 μ M, respectively) and low toxicity on the normal fibroblast cells (IC_{50} > 100 μ M). All of the tested compounds were more potent on T47-D cancer cells and less toxic on NIH-3 T3 normal cells in comparison to reference compound, indibulin. The tubulin polymerization inhibition assay and [³H]colchicine binding assay showed that the main mechanism of cell death by the potent synthesized compounds was not related to an interaction with tubulin. In the annexin V/PI staining assay, the induction of apoptosis in the MCF-7 and MDA-MB231 cell lines was observed. Cell cycle analysis illustrated an increased percentage of sub-G-1 cells in the MDA-MB231 cell line as a further indication of cell death through induction of apoptosis.

Keywords Anti-cancer · Synthesis · MTT · Apoptosis · Indibulin

Introduction

Breast cancer is the most common invasive cancer in women all over the world. Approximately one third (32%) of cancers diagnosed in female patients are breast cancers. It is the fifth most common cause of death related to cancer and the leading cause of cancer death in women. Women have a 1 in 8 lifetime risk of developing breast cancer and 1 in 35 risk of breast cancer causing death in the US [1–4]. The rates of breast cancers are increasing, but these rates differ by race and ethnicity. The disease displays a high level of complexity because of more than 18 sub-types of breast cancer. The stages of tumor progression are not well understood yet in breast cancer.

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Among diverse risk factors concerning developing breast cancer are life style, genetic, environmental and biological factors. There are several strategies to overcome breast cancer including surgery, radiation therapy, chemotherapy, hormonal therapy and targeted therapy, but the morbidity and mortality caused by breast cancer remains high [1, 3–5]. Improving the treatment of breast cancer is thus an important scientific and medical goal.

The microtubule system is an important component of eukaryote cells. It has critical roles in various basic cellular functions, such as formation and maintaining cell morphology, important roles in intracellular and extracellular motility, cell division through formation of the mitotic spindle, organelle transport and cell signaling [6]. During the last decade, attention to anti-tubulin agents and their usage in the treatment of a large number of cancers has been expanded. Microtubule-targeting compounds by affecting microtubule dynamics can cause both the stabilization or destabilization of microtubules, depending on their exact binding site in tubulin, and this disruption of microtubule dynamics invariably lead to cell death through apoptosis. Taxoids and related compounds act in the taxol binding site of tubulin and speed up tubulin polymerization. With a different mechanism of action, inhibition of microtubule assembly, a large number of anti-cancer agents interact with the colchicine site of tubulin. These colchicine site compounds include combrestastatin A-4, nocodazole and indibulin. In both cases, interaction with tubulin disrupts the normal function of cells in mitotic processes and controls the high cell division in cancerous cells [7]. Peripheral neuropathy emerged as a major side effect of paclitaxel and of some anti-mitotic agents that target the colchicine site. Indibulin, representing a new generation of anti-tubulin agent, exerted an interesting anticancer activity without leading to peripheral neuropathy in animal models. In fact, indibulin discriminates between mature neuronal and non-neuronal tubulin [8]. There are reports about efforts for introducing new tubulin polymerization inhibitor inspired by the structure of indibulin, several series of related structures were synthesized and evaluated for anti-cancer activity [9–11] (Fig. 1a). A significant tumor growth inhibition in a mouse xenograft model of head and neck cancer was observed for one of these new indibulin-related structures [9] (Fig. 1b).

Pyrrole is one of the most useful heterocycles in the synthesis of new biologically active molecules. Many compounds that include a pyrrole ring have been synthesized in drug discovery laboratories. Some of these compounds have exerted various biologic effects, including anticancer activity, and these compounds display diverse mechanisms of action [12–14]. From the medicinal chemist's viewpoint, the design and synthesis of new compounds for breast cancer treatment is necessary. We decided that indibulin, with its reported lack of neurotoxicity, was a worthy target, in particular because synthesis of analogues was related to our previous efforts [15–21]

in the design and synthesis of new anticancer agents. We therefore synthesized a novel series of indibulin related diarylpyrrole derivatives and evaluated their anticancer activity on breast cancer cell lines as well as a mouse fibroblast normal cell line to represent noncancer cells, using the 3-(4,5-dimethylthiazoyl-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) cell viability assay. Design of the target compounds was carried out based on development of an indibulin family, in which the fused ring in the indole moiety was converted to a flexible phenyl-pyrrole ring system (Fig. 2). To determine the mechanism of cytotoxic activity of potent compounds, we also investigated the effect of synthesized compounds on tubulin polymerization, colchicine binding, cell cycle progression and apoptosis induction.

Materials and methods

General

All Chemical reagents and solvents were bought from Merck (Darmstadt, Germany). Melting points were determined on a Kofler hot-stage instrument (Reichert, Vienna, Austria). The ^1H NMR spectra were obtained on a Bruker FT-500 MHz spectrometer (Bruker, Darmstadt, Germany) and CDCl_3 was as solvent. ^{13}C NMR spectra were also taken at 125 MHz. The spin multiplicities are written as s (singlet), d (doublet), t (triplet), and m (multiplet) and the Coupling constant (J) values are given in Hertz (Hz). Mass spectra were done on an Agilent 5975B instrument (Agilent Technologies, Santa Clara, CA, USA) with triple-axis detector. IR spectra were obtained by a Nicolet Magna 550-FT spectrometer (Nicolet Instrument Corporation, Madison, WI, USA). Elemental analyses were determined using a Perkin Elmer Model 240-C instrument (PerkinElmer, Hopkinton, MA, USA).

Chemistry

1-(4-chlorophenyl)pentane-1,4-dione (**2a**) and 1-phenylpentane-1,4-dione (**2b**) were Synthesized according to the methods reported in the literature [22, 23].

General procedure for the preparation of 2-methyl-1,5-diaryl-1H-pyrrole derivatives 3a-j

1,4-Diketone derivatives **2a-b** (1 mmol) and the appropriate aniline derivative (1.1 mmol) and *p*-tuloenesulfonic acid (PTSA), catalytic amount, were dissolved and reacted in refluxing ethanol for 6 h. while the reaction is completed, as indicated by thin layer chromatography (TLC), the solvent is evaporated and the remained residue was purified using column chromatography (hexane/ ethyl acetate 10:1 as eluent) to obtain pyrrole derivative **3a-j**.

Fig. 1 **a** Diverse structural changes reported on indibulin structure. **b** Compound A with significant head and neck tumor growth inhibition

General procedure for the preparation of 2-(2-methyl-1,5-diaryl-1*H*-pyrrol-3-yl)-2-oxo-*N*-(pyridin-4-yl)acetamide derivatives 4a-j

After dissolving appropriate pyrrole **3a-j** (1 mmol) and trimethylamine (TEA) (1.2 mmol) in dichloromethane (DCM) (10 mL), the solution was treated with oxalyl chloride (1.1 mmol) dropwise at 0 °C. After the addition was completed, the mixture was stirred at room temperature (RT) for 4 h, then the mixture was concentrated to remove residual oxalyl chloride under reduced pressure. The crude was dissolved in DCM and 4-aminopyridine (1 mmol), TEA (1.2 mmol) and 4-*N,N*-dimethylaminopyridine (DMAP) as a catalyst were added to the reaction mixture. The reaction mixture was

stirred in RT for 12 h and after completion of the reaction (TLC), the solvent was removed using rotary evaporator and the residue was subjected to flash chromatography (hexane/ethyl acetate 3:1, as eluent). Final products **4a-j** were obtained in a pure crystal form.

2-(5-(4-chlorophenyl)-2-methyl-1-phenyl-1*H*-pyrrol-3-yl)-2-oxo-*N*-(pyridin-4-yl)acetamide (4a) Yellow solid; yield 40%; mp 234–236 °C.

IR (KBr, cm^{-1}): 3343, 3150, 3031, 1702, 1638, 1593, 1479, 1415, 1327, 1146, 875, 749, 693; 1H NMR (500 MHz, $CDCl_3$): δ 2.49 (s, 3H, CH_3), 7.00 (d, $J = 8$ Hz, 2H, *H*-Ar), 7.12–7.18 (m, 4H, *H*-Ar), 7.44 (br s, 3H, *H*-Ar), 7.61 (s, 1H, *H*-Pyrrole), 7.65 (d, $J = 5$ Hz, 2H, *H*-Pyridine), 8.58 (d, $J = 5$ Hz, 2H, *H*-Pyridine),

Fig. 2 Structural changes on indibulin in current work

9.37 (s, 1H, NH); ^{13}C NMR (125 MHz, CDCl_3): δ 13.8, 112.8, 113.6, 117.0, 128.1, 128.3, 129.0, 129.4, 129.5, 130.1, 133.0, 134.2, 137.0, 143.8, 144.2, 151.0, 160.9, 180.5; Mass, m/z (%): 415 (M^+ , 10), 294 (35), 133 (16), 103 (50), 73 (100), 51 (5); Anal. Calcd for $\text{C}_{24}\text{H}_{18}\text{ClN}_3\text{O}_2$: C, 69.31; H, 4.36; N, 10.10. Found: C, 69.21; H, 4.31; N, 10.13.

2-(5-(4-chlorophenyl)-1-(4-fluorophenyl)-2-methyl-1H-pyrrol-3-yl)-2-oxo-N-(pyridin-4-yl)acetamide (4b) Yellow solid; yield 43%; mp 204–206 °C.

IR (KBr, cm^{-1}): 3343, 3152, 3034, 1707, 1642, 1595, 1505, 1415, 1326, 1227, 1145, 832, 754, 693; ^1H NMR (500 MHz, CDCl_3): δ 2.47 (s, 3H, CH_3), 6.99 (d, $J = 8$ Hz, 2H, $H\text{-Ar}$), 7.13–7.17 (m, 6H, $H\text{-Ar}$), 7.61 (s, 1H, $H\text{-Pyrrole}$), 7.65 (d, $J = 5.5$ Hz, 2H, $H\text{-Pyridine}$), 8.58 (d, $J = 5.5$ Hz, 2H, $H\text{-Pyridine}$), 9.41 (s, 1H, NH); ^{13}C NMR (125 MHz, CDCl_3): δ 13.7, 112.7, 113.6, 116.6 (d, $J = 22.5$ Hz), 116.8, 128.4, 129.4, 129.8 (d, $J = 8.75$ Hz), 132.9, 133.2, 133.9, 143.8, 144.1, 150.8, 160.8, 162.3 (d, $J = 248.75$ Hz), 180.6; Mass, m/z (%): 433 (M^+ , 12), 312 (100), 248 (7), 128 (8), 51 (5); Anal. Calcd for $\text{C}_{24}\text{H}_{17}\text{ClFN}_3\text{O}_2$: C, 66.44; H, 3.95; N, 9.69. Found: C, 66.58; H, 3.93; N, 9.68.

2-(1,5-bis(4-chlorophenyl)-2-methyl-1H-pyrrol-3-yl)-2-oxo-N-(pyridin-4-yl)acetamide (4c) Yellow solid; yield 44%; mp 213–215 °C.

IR (KBr, cm^{-1}): 3342, 2925, 2854, 1695, 1644, 1592, 1491, 1415, 1326, 1161, 1093, 830, 753, 669; ^1H NMR (500 MHz, CDCl_3): δ 2.48 (s, 3H, CH_3), 7.00 (d, $J = 8$ Hz, 2H, $H\text{-Ar}$), 7.09 (d, $J = 8$ Hz, 2H, $H\text{-Ar}$), 7.17 (d, $J = 8$ Hz, 2H, $H\text{-Ar}$), 7.42 (d, $J = 8$ Hz, 2H, $H\text{-Ar}$), 7.61 (s, 1H, $H\text{-Pyrrole}$), 7.64 (d, $J = 5$ Hz, 2H, $H\text{-Pyridine}$), 8.58 (d, $J = 5$ Hz, 2H, $H\text{-Pyridine}$), 9.36 (s, 1H, NH); ^{13}C NMR (125 MHz, CDCl_3): δ 13.7, 112.9, 113.6, 116.9, 128.5, 129.4, 129.5, 129.8, 131.8, 133.2, 134.0, 135.1, 135.4, 144.0, 144.1, 151.0, 160.9, 180.6; Mass, m/z (%): 449 (M^+ , 7), 328 (60), 149 (25), 100 (90), 72 (100); Anal. Calcd for $\text{C}_{24}\text{H}_{17}\text{Cl}_2\text{N}_3\text{O}_2$: C, 64.01; H, 3.81; N, 9.33. Found: C, 64.19; H, 3.84; N, 9.35.

2-(5-(4-chlorophenyl)-2-methyl-1-(p-tolyl)-1H-pyrrol-3-yl)-2-oxo-N-(pyridin-4-yl)acetamide (4d) Yellow solid; yield 48%; mp 156–158 °C.

IR (KBr, cm^{-1}): 3341, 3144, 3037, 2954, 2852, 1700, 1638, 1593, 1503, 1417, 1326, 1146, 829, 754, 691; ^1H NMR (500 MHz, CDCl_3): δ 2.40 (s, 3H, CH_3), 2.45 (s, 3H, CH_3), 7.00–7.03 (m, 4H, $H\text{-Ar}$), 7.13 (d, $J = 7.5$ Hz, 2H, $H\text{-Ar}$), 7.22 (d, $J = 7.5$ Hz, 2H, $H\text{-Ar}$), 7.60 (s, 1H, $H\text{-Pyrrole}$), 7.65 (d, $J = 5.5$ Hz, 2H, $H\text{-Pyridine}$), 8.57 (d, $J = 5.5$ Hz, 2H, $H\text{-Pyridine}$), 9.45 (s, 1H, NH); ^{13}C NMR (125 MHz, CDCl_3): δ 13.7, 21.1, 112.67, 113.67, 116.65, 127.8, 128.3, 129.3, 130.1, 130.2, 132.8, 133.9, 134.3, 139.0, 143.9, 144.3, 150.9, 161.0, 180.5; Mass, m/z (%): 429 (M^+ , 16), 308 (100), 280 (12), 244 (10), 121 (19), 78 (17), 55 (15); Anal. Calcd for $\text{C}_{25}\text{H}_{20}\text{ClN}_3\text{O}_2$: C, 69.85; H, 4.69; N, 9.77. Found: C, 69.76; H, 4.65; N, 9.79.

2-(5-(4-chlorophenyl)-1-(4-methoxyphenyl)-2-methyl-1H-pyrrol-3-yl)-2-oxo-N-(pyridin-4-yl)acetamide (4e) Yellow solid; yield 51%; mp 162–164 °C.

IR (KBr, cm^{-1}): 3338, 3037, 2962, 2840, 1701, 1638, 1587, 1507, 1416, 1327, 1249, 1030, 832, 755; ^1H NMR (500 MHz, CDCl_3): δ 2.46 (s, 3H, CH_3), 3.84 (s, 3H, OCH_3), 6.93 (d, $J = 8$ Hz, 2H, $H\text{-Ar}$), 7.02 (d, $J = 8$ Hz, 2H, $H\text{-Ar}$), 7.05 (d, $J = 8$ Hz, 2H, $H\text{-Ar}$), 7.15 (d, $J = 8$ Hz, 2H, $H\text{-Ar}$), 7.60 (s, 1H, $H\text{-Pyrrole}$), 7.65 (d, $J = 5$ Hz, 2H, $H\text{-Pyridine}$), 8.58 (d, $J = 5$ Hz, 2H, $H\text{-Pyridine}$), 9.41 (s, 1H, NH); ^{13}C NMR (125 MHz, CDCl_3): δ 13.7, 55.4, 112.57, 113.6, 114.8, 116.5, 128.3, 129.1, 129.4, 129.5, 130.2, 132.9, 134.0, 143.9, 144.5, 150.9, 159.7, 161.0, 180.4; Mass, m/z (%): 445 (M^+ , 17), 324 (90), 135 (31), 84 (100), 51 (34); Anal. Calcd for $\text{C}_{25}\text{H}_{20}\text{ClN}_3\text{O}_3$: C, 67.34; H, 4.52; N, 9.42. Found: C, 67.45; H, 4.50; N, 9.43.

2-(2-methyl-1,5-diphenyl-1H-pyrrol-3-yl)-2-oxo-N-(pyridin-4-yl)acetamide (4f) Yellow solid; yield 54%; mp 216–218 °C.

IR (KBr, cm^{-1}): 3335, 3063, 2855, 1697, 1634, 1582, 1497, 1410, 1328, 1146, 876, 766, 695; ^1H NMR (500 MHz, CDCl_3): δ 2.49 (s, 3H, CH_3), 7.00–7.15 (m, 7H,

H-Ar), 7.41 (br s, 3H, *H*-Ar), 7.64 (br s, 3H, *H*-Pyrrole & *H*-Pyridine), 8.58 (d, $J = 5$ Hz, 2H, *H*-Pyridine), 9.38 (s, 1H, NH); ^{13}C NMR (125 MHz, CDCl_3): δ 13.8, 112.4, 113.6, 116.7, 127.0, 128.1, 128.2, 128.3, 128.8, 129.4, 131.6, 135.2, 137.3, 143.9, 144.1, 150.8, 161.0, 180.6; Mass, m/z (%): 381 (M+, 20), 324 (12), 260 (100), 217 (10), 128 (8), 77 (9); Anal. Calcd for $\text{C}_{24}\text{H}_{19}\text{N}_3\text{O}_2$: C, 75.57; H, 5.02; N, 11.02. Found: C, 75.66; H, 5.05; N, 11.01.

2-(1-(4-fluorophenyl)-2-methyl-5-phenyl-1H-pyrrol-3-yl)-2-oxo-*N*-(pyridin-4-yl)acetamide (4 g) Yellow solid; yield 49%; mp 205–207 °C.

IR (KBr, cm^{-1}): 3336, 3043, 2915, 1701, 1633, 1500, 1477, 1411, 1329, 1145, 966, 875, 766, 695; ^1H NMR (500 MHz, CDCl_3): δ 2.46 (s, 3H, CH_3), 7.06 (d, $J = 7$ Hz, 2H, *H*-Ar), 7.08–7.19 (m, 7H, *H*-Ar), 7.59 (s, 1H, *H*-Pyrrole), 7.65 (d, $J = 5.5$ Hz, 2H, *H*-Pyridine), 8.56 (d, $J = 5.5$ Hz, 2H, *H*-Pyridine), 9.49 (s, 1H, NH); ^{13}C NMR (125 MHz, CDCl_3): δ 13.7, 112.4, 113.6, 116.4 (d, $J = 22.5$ Hz), 116.7, 127.1, 128.1, 128.3, 129.8 (d, $J = 8.75$ Hz), 131.4, 133.1, 135.2, 143.7, 143.9, 150.9, 161.0, 162.2 (d, $J = 248.5$ Hz), 180.7; Mass, m/z (%): 399 (M+, 17), 278 (100), 129 (12), 95 (10), 55 (6); Anal. Calcd for $\text{C}_{24}\text{H}_{18}\text{FN}_3\text{O}_2$: C, 72.17; H, 4.54; N, 10.52. Found: C, 72.10; H, 4.55; N, 10.45.

2-(1-(4-chlorophenyl)-2-methyl-5-phenyl-1H-pyrrol-3-yl)-2-oxo-*N*-(pyridin-4-yl)acetamide (4 h) Yellow solid; yield 46%; mp 215–217 °C.

IR (KBr, cm^{-1}): 3258, 3144, 3058, 2923, 1708, 1648, 1583, 1503, 1412, 1329, 1149, 829, 744, 697; ^1H NMR (500 MHz, CDCl_3): δ 2.47 (s, 3H, CH_3), 7.06–7.12 (m, 4H, *H*-Ar), 7.18–7.22 (m, 3H, *H*-Ar), 7.38 (d, $J = 7.5$ Hz, 2H, *H*-Ar), 7.60 (s, 1H, *H*-Pyrrole), 7.65 (d, $J = 5$ Hz, 2H, *H*-Pyridine), 8.57 (d, $J = 5$ Hz, 2H, *H*-Pyridine), 9.46 (s, 1H, NH); ^{13}C NMR (125 MHz, CDCl_3): δ 13.7, 112.60, 113.64, 116.9, 127.2, 128.2, 128.3, 129.4, 129.6, 131.3, 134.7, 135.1, 135.7, 143.6, 143.9, 150.9, 160.9, 180.7; Mass, m/z (%): 415 (M+, 13), 294 (100), 230 (9), 128 (12), 78 (5); Anal. Calcd for $\text{C}_{24}\text{H}_{18}\text{ClN}_3\text{O}_2$: C, 69.31; H, 4.36; N, 10.10. Found: C, 69.39; H, 4.38; N, 10.14.

2-(2-methyl-5-phenyl-1-(*p*-tolyl)-1H-pyrrol-3-yl)-2-oxo-*N*-(pyridin-4-yl)acetamide (4i) Yellow solid; yield 39%; mp 212–214 °C.

IR (KBr, cm^{-1}): 3347, 3153, 3034, 2919, 2864, 1708, 1637, 1593, 1500, 1409, 1325, 1144, 823, 764, 698; ^1H NMR (500 MHz, CDCl_3): δ 2.38 (s, 3H, CH_3), 2.46 (s, 3H, CH_3), 7.02 (d, $J = 7.5$, 2H, *H*-Ar), 7.07–7.17 (m, 5H, *H*-Ar), 7.20 (d, $J = 7.5$, 2H, *H*-Ar), 7.60 (s, 1H, *H*-Pyrrole), 7.65 (d, $J = 5$ Hz, 2H, *H*-Pyridine), 8.57 (d, $J = 5$ Hz, 2H, *H*-Pyridine), 9.47 (s, 1H, NH); ^{13}C NMR (125 MHz, CDCl_3): δ 13.8, 21.1, 112.2, 113.6, 116.6, 126.9, 127.8, 128.0, 128.2, 129.9, 131.7, 134.5, 135.2, 138.7, 143.9, 144.1, 150.9, 161.1, 180.5; Mass,

m/z (%): 395 (M+, 20), 274 (100), 231 (8), 128 (7), 91 (5); Anal. Calcd for $\text{C}_{25}\text{H}_{21}\text{N}_3\text{O}_2$: C, 75.93; H, 5.35; N, 10.63. Found: C, 75.99; H, 5.38; N, 10.61.

2-(1-(4-methoxyphenyl)-2-methyl-5-phenyl-1H-pyrrol-3-yl)-2-oxo-*N*-(pyridin-4-yl)acetamide (4j) Yellow solid; yield 52%; mp 134–136 °C.

IR (KBr, cm^{-1}): 3338, 3039, 2927, 2837, 1700, 1636, 1594, 1503, 1478, 1411, 833, 754, 694; ^1H NMR (500 MHz, CDCl_3): δ 2.47 (s, 3H, CH_3), 3.83 (s, 3H, OCH_3), 6.91 (d, $J = 8$ Hz, 2H, *H*-Ar), 7.06 (d, $J = 8$ Hz, 2H, *H*-Ar), 7.10 (d, $J = 6$ Hz, 2H, *H*-Ar), 7.12–7.19 (m, 3H, *H*-Ar), 7.61 (s, 1H, *H*-Pyrrole), 7.65 (d, $J = 5$ Hz, 2H, *H*-Pyridine), 8.57 (d, $J = 5$ Hz, 2H, *H*-Pyridine), 9.41 (s, 1H, NH); ^{13}C NMR (125 MHz, CDCl_3): δ 13.8, 55.4, 112.2, 113.6, 114.2, 116.5, 126.9, 128.0, 128.3, 129.1, 129.8, 131.7, 135.3, 143.9, 144.3, 150.9, 159.5, 161.1, 180.5; Mass, m/z (%): 411 (M+, 19), 290 (100), 236 (25), 152 (15), 111 (30), 83 (51), 57 (60); Anal. Calcd for $\text{C}_{25}\text{H}_{21}\text{N}_3\text{O}_3$: C, 72.98; H, 5.14; N, 10.21. Found: C, 72.87; H, 5.10; N, 10.23.

Biological evaluation

Cell culture

For cell culture, appropriate cell lines, MCF-7, MDA-MB231, T47-D (human breast cancer cell lines) and NIH-3 T3 (mouse fibroblast cell line) were purchased from the Pasteur Institute (Tehran, Iran). RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) was used for cell culturing, supplemented with penicillin (100 U/mL), 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), and (100 $\mu\text{g/mL}$) streptomycin (Roche, Mannheim, Germany) in a humidified incubator with 5% CO_2 at 37 °C.

Cytotoxicity evaluation by the MTT assay

MTT assay for all compounds was done according to method reported in our previous work [24].

Colchicine binding inhibition and in vitro inhibitory tubulin polymerization assay

In the present study, purified bovine brain tubulin was used in the tubulin polymerization and colchicine binding assays, prepared as reported previously [25]. The experimental method for measuring [^3H]colchicine binding inhibition to tubulin was reported in detail previously [26]. Briefly, the ligand binding assay was performed in 0.1 mL reaction volumes. Each reaction mixture contained 0.1 mg/mL (1.0 μM) tubulin, 5.0 μM [^3H]colchicine and 5.0 μM of potential inhibitory compound. Combretastatin-A4 (CA-4), a highly potent inhibitor of colchicine binding to tubulin, was used as a positive

Scheme 1 Synthetic pathway of desired compounds **4a-j**

standard [27]. Reaction mixtures were incubated for 10 min at 37 °C, the time that the reaction in control reaction mixtures is about 40–60% complete. At this time, reaction mixtures were diluted and stopped with water at 0 °C, and the mixtures were filtered through Whatman DEAE-cellulose filters. The filters were then washed with ice-cold water. Radiolabel bound to the filters was measured using a liquid scintillation counter. For investigation of tubulin polymerization inhibitory potential of selected compounds, 1.0 mg/mL (10 μM) purified tubulin was treated without Guanosine-5'-triphosphate (GTP) with various concentrations of synthesized compound at 30 °C for 15 min. Then reaction mixtures were placed on ice, and GTP (10 μL of 10 μM) was added. Reaction mixtures were transferred to cuvettes held at 0 °C in recording spectrophotometers. After baselines were recorded at 350 nm at 0 °C, the temperature was increased to 30 °C rapidly (over about 30 s) and change in turbidity was followed over 20 min. Compound concentrations that resulted in a 50% decrease in turbidity (IC₅₀) were then determined [28].

Annexin V-FITC/PI assay

To quantify the cell death modality, the annexin V/propidium iodide (PI) staining assay was used. Briefly, 3 × 10⁵ cells (MCF-7 for **4b** and MDA-MB231 for **4e**) were plated and then the cells were treated with **4b** or **4e** (at the IC₅₀

concentration for 24 h). After treatment, cells were washed twice with phosphate buffered saline (PBS), mixed with 500 μL of binding buffer, and stained with 5 μL of annexin V-fluorescein isothiocyanate (FITC) and 5 μL of PI (PI 50 μg/mL) for 10 min at RT in the dark. Apoptotic cells were quantified by a FACS Calibur flow cytometer (BD Biosciences,

Table 1 In vitro cytotoxic activities (IC₅₀)^{a, b} of synthesized compounds **4a-j**

Compounds	MCF-7	T47-D	MDA-MB231	NIH-3 T3
4a	>10	34.04 ± 2.9	33.79 ± 1.8	99.1 ± 1.4
4b	7.5 ± 0.5	41.95 ± 2.1	33.63 ± 3.2	21.0 ± 2.3
4c	>10	40.56 ± 3.6	37.11 ± 2.8	76.2 ± 3.5
4d	>10	43.28 ± 1.7	28.69 ± 1.6	53.5 ± 1.7
4e	>10	45.49 ± 2.3	10.55 ± 1.3	47.4 ± 1.9
4f	>10	16.6 ± 1.5	11.82 ± 2.2	>100
4g	>10	15.61 ± 2.1	13.33 ± 1.5	>100
4h	>10	13.49 ± 2.7	26.02 ± 3.1	35.5 ± 2.8
4i	>10	12.19 ± 1.4	28.06 ± 3.3	37.9 ± 1.8
4j	>10	23.17 ± 1.9	32.52 ± 2.4	49.3 ± 2.1
Indibulin	>100	>100	9.0 ± 0.6	11.2 ± 1.6

^a IC₅₀ values are in μM

^b IC₅₀, compound concentration required to inhibit cell proliferation by 50%

San Jose, CA); 1×10^4 cells were counted for each sample. Both early apoptotic (annexin V-positive, PI-negative) and late apoptotic (double positive of annexin V and PI) cells were detected. The percentages of cells in each quadrant were analyzed using the Flowing Software version 2.4.1 (Turku Centre for Biotechnology University of Turku, Finland). The experiments were repeated at least three times.

Flow cytometric analysis of cell cycle distribution

For analysis of DNA content using flow cytometry, 1×10^6 MCF-7 cells, in a 6-well plate, were treated with compound **4e** (at the IC_{50} concentration) for 24 h. For cell fixation, cells were centrifuged and 0.7 ml of cold 75% ethanol was added to samples, and the samples were placed on ice for at least 3 h. After washing with PBS, cells were resuspended in 0.25 mL of PBS, with 5 μ L of 10 mg/mL RNase A and Triton X-100 (0.1%). Then, cells were incubated at 37 °C for 1 h, and 10 μ L

of 50 μ g/mL PI was added. The fluorescence of the cells was determined with a FACS-Calibur flow cytometer (BDBiosciences, San Jose, CA) [29].

Results and discussion

Chemistry

The synthetic pathway of novel target compounds is outlined in Scheme 1. In the first step, appropriate derivatives of benzaldehyde, **1a** or **1b**, were treated with methyl vinyl ketone in the presence of sodium cyanide as catalyst and *N,N*-dimethylformamide (DMF) as solvent to yield 1 aryl-1,4-pentanediones **2a-b**. Then, diones **2a-b** were refluxed with aniline derivatives using a catalytic amount of PTSA in ethanol for 6 h to give corresponding 1,5-diarylpyrrole derivatives **3a-j**. The compounds **4a-j** were prepared through two continuous

Fig. 3 **4b** induced cell death modes in the MCF-7 cell line. **a** **4b** (7.5 μ M) treated MCF-7 cells for 24 h. **b** After 24 h of incubation with **4b**, both the percentages of early apoptotic cells and late apoptotic cells (stained with both annexin V and PI) increased significantly. Data are representative of three independent experiments. * $P < 0.05$ denotes a mean significantly different from untreated cells. **4e** induced cell death modalities in the MDA-MB-231 cell line. **c** **4e** (10.55 μ M) treated MDA-MB-231 for 24 h. **d** After 24 h of incubation with **4e**, the percentages of early and late apoptotic cells (stained with both annexin V and PI) increased significantly. Data are representative of three independent experiments. * $P < 0.05$ denotes a mean significantly different from untreated cells

Fig. 3 (continued)

reactions. In the first step, reaction of oxalyl chloride with pyrrole derivatives in the presence of dry TEA in DCM at RT gave the related acyl chloride. Then, the mixture was concentrated under reduced pressure to remove excess oxalyl chloride. The residue

was dissolved in dry DCM and TEA, a catalytic amount of DMAP and 3-aminopyridine were added and stirred at RT for 12 h. The mixture was concentrated and the final products **4a-j** were purified using column chromatography (hexane/ethyl

Fig. 4 **4e** induces cell cycle arrest in the MDA-MB-231 cells. **a** Typical DNA content histograms of cells treated with **4e** (10.55 μ M) for 24 h

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Cell cycle analysis

With regard to cytotoxic activity and apoptosis induction ability of **4e**, we evaluated the effect of **4e** on cell cycle distribution of cultured MDA-MB231 cell line using flow cytometric cell cycle analysis. After treatment of cells with compound **4e** at the IC_{50} concentration for 24 h, there was an increased percentage of cells in the sub-G1 region, representing an increase in cell death consistent with the induction of apoptosis shown in Fig. 4.

Docking study

With respect to the results of tubulin polymerization inhibition, we decided to study the possible mode of interactions of **4f** and **4g** with tubulin protein using molecular docking software to understand the reason of inactivity of synthesized compounds in inhibiting the tubulin polymerization. Indibulin was also docked in tubulin active site for comparison as a reference. Conversion of rigid and flat indole part of indibulin to flexible 2-arylpyrrole and substituting the N-4-chlorobenzyl part with N-aryl moiety, drastically decreased the effect of synthesized compounds on tubulin polymerization in comparison with indibulin, and the interaction of these parts with active site of tubulin is the key cause in showing activity or inactivity of tested compounds on tubulin polymerization. In both **4f** and **4g**, phenyl ring attached to C-5 of pyrrole ring showed interactions with VAL176, SER177 and TYR223 residues. These interactions exactly repeated between the above residues and six-membered ring of indole of indibulin. SER177 has another interaction with five-membered ring of indole of indibulin, which this interaction is absent between SER177 and pyrrole ring of **4f** and **4g**. Because of the ability of phenyl ring attached to C-5 of pyrrole ring in **4f** and **4g**, it seems the interactions of the phenyl ring in these compounds is not as strong as the interactions in indole of indibulin with the mentioned residues. The N-4-chlorobenzyl moiety of indibulin has interactions with ALA787, LEU681 and CYS674, but these interactions are completely absent for the N-aryl part of the **4f** and **4g**. There is also some other differences in mode of interactions between the residues in active site and structures of **4f**, **4g** and indibulin. Altogether, it seems these differences in interactions, especially presented for N-4-chlorobenzyl moiety of indibulin, are the cause of inactivity of **4f** and **4g** in tubulin polymerization inhibition (Fig. 5).

Conclusions

We synthesized a novel series of indibulin-related 1,5-diarylpyrroles as potential antiproliferative agents and investigated their cytotoxic activity against four diverse cell lines.

Compounds **4b** and **4e** showed the highest cytotoxic activity on the MCF-7 and MDA-MB231 cell lines, respectively. Both of the compounds induced apoptosis in tested cell lines. Compounds **4f** and **4g** not only showed high toxicity on the MDA-MB231 cancer cell line but also showed very low toxicity on normal cells (IC_{50} above 100 μ M). This is an ideal choice to continue further pharmacological investigation on these two derivatives for finding their exact mechanism of action. A ligand binding assay plus a tubulin polymerization inhibitory assay showed that interaction of active compounds with tubulin was not the cause of the anticancer activity by the synthesized compounds and that other important factors may trigger apoptosis induction and finally cell death. Our data indicate that it will be beneficial to investigate other derivatives with the 1,5-diaryl pyrrole scaffold in a search for more potent and safer anticancer agents.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Santos CCF, Paradelo LS, Novaes LFT, Dias SMG, Pastre JC. Design and synthesis of cenocladamide analogues and their evaluation against breast cancer cell lines. *Med Chem Commun*. 2017;8: 755–66.
2. Yao N, Chen CY, Wu CY, Motonishi K, Kung HJ, Lam KS. Novel flavonoids with antiproliferative activities against breast cancer cells. *J Med Chem*. 2011;54:4339–49.
3. Ananda Kumar CS, Nanjunda Swamy S, Thimmegowda NR, Benaka Prasad SB, Yip GW, Rangappa KS. Synthesis and evaluation of 1-benzhydryl-sulfonylpiperazine derivatives as inhibitors of MDA-MB-231 human breast cancer cell proliferation. *Med Chem Res*. 2007;16:179–87.
4. Gautam Y, Dwivedi S, Srivastava A, Hamidullah H, Singh A, Chanda D, et al. 2-(3', 4'-dimethoxybenzylidene) tetralone induces anti-breast cancer activity through microtubule stabilization and activation of reactive oxygen species. *RSC Adv*. 2016;6:33369–79.
5. Bass R, Jenkinson S, Wright J, Srinivasan TS, Marshall JC, Castagnolo D. Synthesis and biological evaluation of novel amidinourea and triazine congeners as inhibitors of MDA-MB-231 human breast cancer cell proliferation. *Chem Med Chem*. 2017;12:288–91.
6. Romagnoli R, Baraldi PG, Salvador MK, Preti D, Tabrizi MA, Brancale A, et al. Synthesis and evaluation of 1,5-disubstituted tetrazoles as rigid analogues of combretastatin A-4 with potent antiproliferative and antitumor activity. *J Med Chem*. 2012;55:475–88.
7. Lu Y, Chen J, Xiao M, Li W, Miller DD. An overview of tubulin inhibitors that interact with the colchicine binding site. *Pharm Res*. 2012;29:2943–71.

8. Wienecke A, Bacher G. Indibulin, a novel microtubule inhibitor, discriminates between mature neuronal and nonneuronal tubulin. *Cancer Res.* 2009;69:171–7.
9. Colley HE, Muthana M, Danson SJ, Jackson LV, Brett ML, Harrison J, et al. An orally bioavailable, indole-3-glyoxylamide based series of tubulin polymerization inhibitors showing tumor growth inhibition in a mouse xenograft model of head and neck cancer. *J Med Chem.* 2015;58:9309–33.
10. Marchand P, Antoine M, Le Baut G, Czech M, Baasner S, Günther E. Synthesis and structure–activity relationships of N-aryl(indol-3-yl)glyoxamides as antitumor agents. *Bioorg Med Chem.* 2009;17: 6715–27.
11. Li WT, Hwang DR, Chen CP, Shen CW, Huang CL, Chen TW, et al. Synthesis and biological evaluation of N-heterocyclic indolyl glyoxylamides as orally active anticancer agents. *J Med Chem.* 2003;46:1706–15.
12. Fang Z, Liao PC, Yang YL, Yang FL, Chen YL, Lam Y, et al. Synthesis and biological evaluation of polyenylpyrrole derivatives as anticancer agents acting through caspases-dependent apoptosis. *J Med Chem.* 2010;53:7967–78.
13. Carbone A, Pennati M, Parrino B, Lopercolo A, Baraja P, Montalbano A, et al. Novel 1h-pyrrolo[2,3-b]pyridine derivative nortoposetin analogues: synthesis and antitumor activity in peritoneal mesothelioma experimental models. *J Med Chem.* 2013;56:7060–72.
14. Wang S, Wood G, Meades C, Griffiths G, Midgley C, McNae I, et al. Synthesis and biological activity of 2-anilino-4-(1H-pyrrol-3-yl) pyrimidine CDK inhibitors. *Bioorg Med Chem Lett.* 2004;14: 4237–40.
15. Ghasemi M, Ghadbeighi S, Amirhamzeh A, Tabatabai SA, Ostad SN, Shafiee A, et al. Synthesis, molecular docking study, and cytotoxic activity of 1,3,5-triaryl pyrazole derivatives. *Lett Drug Des Discov.* 2016;13:121–8.
16. Ghadbeighi S, Ostad SN, Shafiee A, Amini M. Synthesis and anticancer activity of 1,3,5-triaryl-1H-pyrazole. *Lett Drug Des Discov.* 2015;12:754–9.
17. Miralinaghi P, Salimi M, Amirhamzeh A, Norouzi M, Kandelousi HM, Shafiee A, et al. Synthesis, molecular docking study, and anticancer activity of triaryl-1,2,4-oxadiazole. *Med Chem Res.* 2013;22:4253–62.
18. Salehi M, Ostad SN, Riazi GH, Assadieskandar A, Shavi TC, Shafiee A, et al. Synthesis, cytotoxic evaluation, and molecular docking study of 4,5-diaryl-thiazole-2-thione analogs of combretastatin A-4 as microtubule-binding agents. *Med Chem Res.* 2014;23:1465–73.
19. Zareian B, Ghadbeighi S, Amirhamzeh A, Ostad SN, Shafiee A, Amini M. Synthesis, molecular docking study, and cytotoxic activity of 3,4-diaryl-5-(4-pyridinyl)-1,2,4-oxadiazole. *Med Chem.* 2016;12:394–401.
20. Saravani F, Saeedian Moghadam E, Salehabadi H, Ostad SN, Pirali Hamedani M, Amanlou M, et al. Synthesis, anti-proliferative evaluation, and molecular docking studies of 3-(alkylthio)-5,6-diaryl-1,2,4-triazines as tubulin polymerization inhibitors. *Lett Drug Des Discov.* 2018;15. <https://doi.org/10.2174/1570180815666180727114216>.
21. Saeedian Moghadam E, Saravani F, Ostad SN, Tavajohi S, Pirali Hamedani M, Amini M. Design, synthesis and cytotoxicity evaluation of indibulin analogs. *Heterocycl Commun.* 2018;24:211–8.
22. Peloquin AJ, Stone RL, Avila SE, Rudico ER, Hom CB, Gardner KA, et al. Synthesis of 1,3-diphenyl-6-alkyl/aryl-substituted fulvene chromophores: observation of π - π interactions in a 6-pyrene-substituted 1, 3-diphenylfulvene. *J Org Chem.* 2012;77:6371–6.
23. Stetter H. Addition von aromatischen und heterocyclischen Aldehyden an α , β unsaturated ketone. *Chem Ber.* 1974;107:2453–8.
24. Saeedian Moghadam E, Amini M. 2-[2-Methyl-5-phenyl-1-(3,4,5-trimethoxyphenyl) 1Hpyrrol-3-yl]-2-oxo-N-(pyridin-4-yl) acetamide. *Molbank.* 2018;2018. <https://doi.org/10.3390/M1002>.
25. Hamel E, Lin CM. Separation of active tubulin and microtubule-associated proteins by ultracentrifugation and isolation of a component causing the formation of microtubule bundles. *Biochem.* 1981;23:4173–84.
26. Hamel E, Lin CM. Stabilization of the colchicine-binding activity of tubulin by organic acids. *Biochim Biophys Acta.* 1984;675:226–31.
27. Verdier-Pinard P, Lai JY, Yoo HD, Yu J, Marquez B, Nagle DG, et al. Structure-activity analysis of the interaction of Curacin a, the potent colchicine site antimitotic agent, with tubulin and effects of analogs on the growth of MCF-7 breast cancer cells. *Mol Pharmacol.* 1998;53:62–76.
28. Hamel E. Evaluation of antimitotic agents by quantitative comparisons of their effects on the polymerization of purified tubulin. *Cell Biochem Biophys.* 2003;38:1–22.
29. Assadieskandar A, Amini M, Ostad SN, Riazi GH, Shavi TC, Shafiee B, et al. Design, synthesis, cytotoxic evaluation and tubulin inhibitory activity of 4-aryl-5-(3,4,5-trimethoxyphenyl)-2-alkylthio-1H-imidazole derivatives. *Bioorg Med Chem.* 2013;21: 2703–9.
30. Bacher G, Nickel B, Emig P, Vanhoefer U, Seeber S, Shandra A, et al. D-24851, a novel synthetic microtubule inhibitor, exerts curative antitumoral activity in vivo, shows efficacy toward multidrug-resistant tumor cells, and lacks neurotoxicity. *Cancer Res.* 2001;61:392–9.

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