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RESEARCH ARTICLE

Synthesis of 1-acetyl-3-(2-thienyl)-5-aryl-2-pyrazoline derivatives and evaluation of their anticancer activity

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Abstract

In the present study, 1-acetyl-3-(2-thienyl)-5-aryl-2-pyrazoline derivatives (**1**–**6**) were synthesized via the ring closure reaction of 1-(2-thienyl)-3-aryl-2-propen-1-ones with hydrazine hydrate in acetic acid. The chemical structures of the compounds were elucidated by IR, ^{1}H-NMR, ^{13}C-NMR and mass spectral data and elemental analyses. MTT assay, analysis of DNA synthesis and caspase-3 activation assay were carried out to determine anticancer effects of the compounds on A549 and C6 cancer cell lines. They exhibited dose-dependent anticancer activity against A549 and C6 cancer cell lines. Anticancer activity screening results revealed that compounds **1**, **2** and **4** were the most potent derivatives among these compounds. But anticancer effects of these compounds may result from different death mechanisms in A549 and C6 cell lines.

Keywords: Pyrazoline, anticancer activity, MTT assay, caspase-3 activation

Introduction

Cancer, which is characterized by the uncontrolled growth of abnormal cells in the body, has emerged as the second leading cause of death throughout the world after cardiovascular disorders. The incidence of cancer has increased dramatically in the last decades. As a consequence of this situation, the treatment of cancer has gained great importance[1–3].

Chemotherapy, which is used to destroy cancer cells with drugs, is one of the most widely used treatment options in cancer treatment. Although there are many anticancer drugs currently available, their use in the treatment is limited due to their adverse effects, and the development of resistance. The side effects accompanying the use of these drugs arise from the fact that anticancer agents act on both tumour cells and healthy cells[3–6].

From the above discussion, it is clear that the search for new effective compounds which can selectively inhibit the proliferation of abnormal cells only with least or no affect on normal cells has gained great importance[6].

Over the past two decades, pyrazolines have attracted a great deal of interest as privileged scaffolds owing to their synthetic and biological importance in medicinal chemistry. Pyrazoline derivatives have been reported to exhibit a wide spectrum of biological effects including anticancer activity[6–16]. Some compounds bearing pyrazole moiety exert their therapeutic action by inhibiting different types of enzymes that play important roles in cell division[16–18].

Manna et al. synthesized 1-acetyl-3,5-diaryl-4,5-dihydro-(1H)-pyrazole derivatives and investigated their anticancer activity and affinity binding to P-glycoprotein. In the same study, some compounds were found to bind to P-glycoprotein with greater affinity[19].

Many researchers have also studied thiophenes extensively due to the fact that new effective compounds can be obtained by the bioisosteric replacement of benzene ring with thiophene[20,21]. Some studies have confirmed that compounds bearing thiophene moiety possess antitumor activity[22–24].

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On the basis of these findings, we became interested in biological evaluation of pyrazoline derivatives as antitumor agents. Herein, we described the synthesis of new pyrazoline derivatives bearing thiophene moiety and focused on their potential anticancer effects against A549 and C6 cancer cell lines using MTT assay. Among these derivatives, the most effective compounds were evaluated for their DNA synthesis inhibitory activity and effects on caspase-3 activation.

Experimental

Chemistry

All reagents were used as purchased from commercial suppliers without further purification. Melting points were determined by using an Electrothermal 9100 digital melting point apparatus and were uncorrected (Electrothermal, Essex, UK). The compounds were checked for purity by TLC on silica gel 60 F254. Spectroscopic data were recorded on the following instruments: IR, Shimadzu 435 IR spectrophotometer (Shimadzu, Tokyo, Japan); ^{1}H-NMR, Bruker 500 MHz NMR spectrometer (Bruker Bioscience, Billerica, MA, USA) and ^{13}C-NMR, Bruker Avance II 125 MHz NMR spectrometer (Bruker Bioscience, Billerica, MA, USA) in DMSO-d_6 using TMS as internal standard; MS-FAB, VG Quattro mass spectrometer (Fisons Instruments Vertriebs GmbH, Mainz, Germany), Elemental analyses were performed on a Perkin Elmer EAL 240 elemental analyser (Perkin Elmer, Norwalk, CT, USA).

General procedure for the synthesis of compounds

1-(2-Thienyl)-3-aryl-2-propen-1-ones (Chalcones)

A mixture of 2-acetylthiophene (0.04 mol), aromatic aldehyde (0.04 mol) and 10% aqueous sodium hydroxide (10 mL) in ethanol (50 mL) was stirred at room temperature for 4 h. The resulting solid was washed, dried and crystallized from ethanol[26].

1-Acetyl-3-(2-thienyl)-5-aryl-2-pyrazoline derivatives (1–6)

To a solution of chalcone derivative (1 mmol) in acetic acid (3 mL), hydrazine hydrate (80%) (0.3 mL, 6 mmol) was added. The mixture was refluxed under stirring for 5 h, and then poured onto crushed ice. The precipitate was filtered off, washed with cold water, and crystallized from methanol to give pyrazolines[9].

1-Acetyl-3-(2-thienyl)-5-(4-cyanophenyl)-2-pyrazoline (5)

IR [υ, cm^{-1}, KBr]: 1648 (C=O), 1597 (C=N), 827 (1,4-disubstituted benzene C-H).

^{1}H-NMR (500 MHz, DMSO-d_6): δ 2.27 (3H, s, $COCH_3$), 3.16 (1H, dd J_{AM} = 17.90 Hz, J_{AX} = 4.89 Hz, C_4-H_A of pyrazoline ring), 3.92 (1H, dd J_{MA} = 17.95 Hz, J_{MX} = 11.96 Hz, C_4–H_M of pyrazoline ring), 5.66 (1H, dd J_{MX} = 11.88 Hz, J_{AX} = 4.87 Hz, C_5-H_X of pyrazoline ring), 6.78–7.76 (7H, m, aromatic protons).

^{13}C-NMR (125 MHz, DMSO-d_6): δ 22.04 (CH_3), 43.36 (CH_2), 59.76 (CH), 124.71 (C), 125.89 (CH), 127.14 (CH), 129.04 (2CH), 130.20 (CH), 133.22 (CH), 136.37 (CH), 137.17 (C), 138.44 (C), 139.61 (C), 150.57 (C), 167.23 (C).

MS-FAB$^+$: *m/z*: 296 [M + 1]. For $C_{16}H_{13}N_3OS$ calculated: 65.06 % C, 4.44 % H, 14.23 % N; found: 64.88 % C, 4.31 % H, 14.29 % N.

1-Acetyl-3-(2-thienyl)-5-(4-isopropylphenyl)-2-pyrazoline (6)

IR [υ, cm^{-1}, KBr]: 1648 (C=O), 1588 (C=N), 820 (1,4-disubstituted benzene C-H).

^{1}H-NMR (500 MHz, DMSO-d_6): δ 1.18 (6H, s, $2CH_3$), 2.26 (3H, s, $COCH_3$), 2.87 (1H, m, $CH(CH_3)_2$), 3.17 (1H, dd J_{AM} = 17.73 Hz, J_{AX} = 4.18 Hz, C_4-H_A of pyrazoline ring), 3.84 (1H, dd J_{MA} = 17.74 Hz, J_{MX} = 11.80 Hz, C_4–H_M of pyrazoline ring), 5.53 (1H, dd J_{MX} = 11.61 Hz, J_{AX} = 4.14 Hz, C_5–H_X of pyrazoline ring), 7.09–7.45 (5H, m, aromatic protons), 7.75 (2H, d J = 5.03 Hz, phenyl protons).

^{13}C-NMR (125 MHz, DMSO-d_6): δ 22.16 (CH_3), 24.32 ($2CH_3$), 34.22 (CH), 43.23 (CH_2), 59.75 (CH), 125.81 (CH), 128.53 (CH), 129.67 (2CH), 130.81 (CH), 134.62 (CH), 136.86 (CH), 137.21 (C), 138.72 (C), 139.73 (C), 150.64 (C), 167.38 (C).

MS-FAB$^+$: *m/z*: 313 [M + 1]. For $C_{18}H_{20}N_2OS$ calculated: 69.20 % C, 6.45 % H, 8.97 % N; found: 69.28 % C, 6.53 % H, 8.95 % N.

Biochemistry

Cell culture and drug treatment

C6 glioma cells were incubated in Dulbecco's modified Eagle's medium (DMEM) (Sigma, Deisenhofen, Germany) supplemented with 10% fetal calf serum (Gibco, Paisley, Scotland). A549 cells were incubated in 90% RPMI supplemented with 10% fetal bovine serum (Gibco, Paisley, Scotland). All media were supplemented with 100 IU/mL penicillin-streptomycin (Gibco, Paisley, Scotland) and cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Exponentially growing cells were plated at 2×10^4 cells/mL into 96-well microtiter tissue culture plates (Nunc, Denmark) and incubated for 24 h before the addition of the drugs (the optimum cell number for cytotoxicity assays was determined in preliminary experiments). Stock solutions of compounds were prepared in dimethyl sulphoxide (DMSO; Sigma Aldrich, Poole, UK) and further dilutions were made with fresh culture medium (the concentration of DMSO in the final culture medium was <0.1% which had no effect on the cell viability).

MTT assay for cytotoxicity of compounds

The level of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) reduction was quantified as previously described in the literature with small modifications[27,28]. After 24 h of preincubation, the tested compounds were added to give final concentration in the range 3.9–500 μg/mL and the cells were incubated for 24 h. At the end of this period, MTT was added to a final concentration of 0.5 mg/mL and the cells were incubated for 4 h at 37°C. After the medium was removed, the formazan crystals formed by

MTT metabolism were solubilized by addition of 200 µL DMSO to each well and absorbance was read at 540 nm with a microtitre plate spectrophotometer (Bio-Tek plate reader). Every concentration was repeated in three wells and IC_{50} values were defined as the drug concentrations that reduced absorbance to 50% of control values.

Analysis of DNA synthesis

Analysis of DNA synthesis was measured by Roche Cell Proliferation ELISA, BrdU (colorimetric) kit. This immunostaining procedure is based on measuring the incorporation of bromodeoxyuridine (BrdU) into nuclear DNA in place of thymidine during the S-phase of the cell cycle using specific anti-BrdU antibodies[29]. Hence, such method provides a colorimetric measurement for DNA synthesis inhibition ratio of the carcinogenic cells. Firstly cells were seeded into 96-well flat-bottomed microtiter plates at a density of 2×10^3. The tumor cells were cultured in the presence of various doses of compounds **1**, **2**, **4** and mitoxantrone. Microtiter plates were incubated at 37°C in a 5% CO_2/95% air humidified atmosphere for 24 h. Cells were labeled with 10 µL BrdU solution for 2 h and then fixed. Anti-BrdU-POD (100 µL) was added and incubated for 90 min. Finally, wells were washed with BPS and cells were incubated with substrate. Absorbance of the samples was measured with an ELX808-IU Bio-Tek apparatus at 492 nm. All experiments were repeated two times. For each compound dose, duplicate wells were used.

Spectrofluorometric analysis of Caspase-3 activation

Caspase-3 activation was analysed by Spectrofluorometric Caspase-3 Assay kit (BD Pharmingen). This kit is designed to measure caspase-3 or DEVD-cleaving activity, an early marker of cells undergoing apoptosis[30]. Firstly cells (1–10^6 cells/mL) were washed with PBS and resuspended in cold cell lysis buffer and incubated for 30 min on ice. Then cell lysates were prepared after 24 h incubation with compounds **1**, **2**, **4** and mitoxantrone. For each reaction, 5 µL reconstituted Ac-DEVD-AMC (synthetic tetrapeptide fluorogenic substrate for Caspase-3) was added to a well containing 0.2 mL of 1 X HEPES buffer. 20 µL cell lysate was added to each well/reaction. Reaction mixtures were incubated for 1 h at 37°C. The amount of AMC liberated from Ac-DEVD-AMC was measured using plate reader (Perkin Elmer/Victor/X3) with an excitation wavelenght of 380 nm and an emmision wavelength of 460 nm. Apoptotic cell lysates containing active Caspase-3 yield a considerable emission as compared to controls. Non-apoptotic control cell lysates AMC emission was accepted as 100% and other cell lysates emissions were measured according to control cells emissions. All experiments were repeated two times. For each compound dose, duplicate wells were used.

Acridine orange/ethidium bromide staining methods

C6 glioma cells were cultured on cover slips at 1×10^5 cells/well onto 6 wells plate until 50% confluent. The medium was replaced with fresh medium containing different concentrations of compounds **1**, **2**, **4** and mitoxantrone. The cells, without treatment, were used as negative controls. Cells then were incubated for 24 h in humidified atmosphere at 37°C in 5% CO_2. The medium was removed and cells were washed three times with PBS. Then cells were fixed with 70% ethanol and incubated for 5 min in RT. Cells were washed with PBS. Then working Solution, ethidium bromide/acridin orange (a mixture of acridine orange and ethidium bromide 1:1, 100 µg/mL) was added and incubated for 5 min. After cells were washed with PBS, cover slip, containing cells was removed and covered on the object glass. Then assessment can be carried out under fluorescence microscope (Olympus)[31].

Acridine Orange/Ethidium Bromide combination was used to visualize cells with aberrant chromatin organization. Acridine Orange was used to visualize the number of cells which has undergone apoptosis. The differential uptake of these two dyes allows the identification of viable and non-viable cells[32].

Statistical analyses

Statistical Package for the Social Science (SPSS) for Windows 15.0 was used for statistical analysis. Data was expressed as Mean ± SD. Comparisons were performed by one way ANOVA test for normally distributed continuous variables and post hoc analyses of group differences were expressed by the Tukey test.

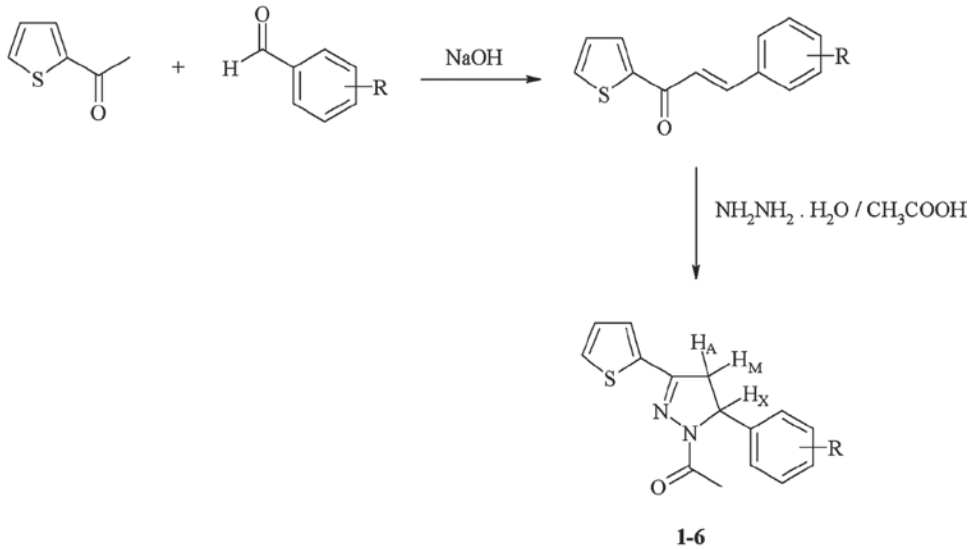
Results and discussion

The synthesis of pyrazoline derivatives (**1–6**) was carried out according to the steps shown in Scheme 1. In the initial step, 1-(2-thienyl)-3-aryl-2-propen-1-ones were synthesized via the base-catalyzed Claisen-Schmidt condensation of 2-acetylthiophene with appropriate aldehydes. The ring closure reaction of chalcones with hydrazine hydrate in acetic acid afforded 1-acetyl-3-(2-thienyl)-5-aryl-2-pyrazolines (**1–6**). Some properties of the compounds were given in Supplementary Table S1.

The structures of all compounds were confirmed by IR, ^{1}H-NMR, ^{13}C-NMR, mass spectral data and elemental analyses. The IR data were very informative and provided evidence for the formation of the expected structures. C=O, C=N, and 1,4-disubstituted benzene C-H functions absorbed strongly in the expected regions: C=O at 1640–1655 cm^{-1}, C=N at 1614-1402 cm^{-1} and 1,4-disubstituted benzene C-H at 820–830 cm^{-1}, respectively. The ^{1}H-NMR spectral data were also consistent with the assigned structures. In the ^{1}H-NMR spectra of all compounds, the CH_2 protons of the pyrazoline ring resonated as a pair of doublets of doublets at δ = 3.10–3.17, and 3.81–3.92 ppm. The CH proton appeared as doublet of doublets at δ = 5.45–5.66 ppm due to vicinal coupling with two magnetically non-equivalent protons of the methylene group at position 4 of the pyrazoline ring (J_{AM} = 17.71–17.95 Hz, J_{AX} = 4.12–4.89 Hz, J_{MX} = 11.61–11.96 Hz). Moreover, the methyl protons of the acetyl group

appeared as a singlet peak at δ = 2.23–2.27 ppm. All the other aromatic and aliphatic protons were observed at expected regions. The signals obtained from ^{13}C-NMR spectra further confirmed the proposed structures; the C_4 and C_5 carbons of the pyrazoline ring resonated at 43.23–43.56 and 59.49–59.96 ppm, respectively. All compounds showed a signal at 150.57–150.68 ppm, which was assignable to azomethine carbon of pyrazoline ring. In the ^{13}C-NMR spectra of the compounds, the acetyl carbon was observed in the region 167.16–167.57 ppm. All compounds gave satisfactory elemental analysis. The mass spectra (MS-FAB$^+$) of all compounds showed [M + 1] peaks, in agreement with their molecular formula. The spectral data of compounds **1**, **2**, **3** and **4** were given in Supplementary Material.

Anticancer activity screening of the compounds against A549 and C6 cell lines was carried out at four steps. In the first step, dose-dependent cytotoxic effects of the compounds were tested on A549 and C6 cancer cell lines using MTT assay. Then, DNA synthesis inhibition assay



1-6

1: R= 4-CH_3, **2:** R= 4-Br, **3:** R= 4-OH, **4:** R= 3,4-OCH_2O, **5:** R= 4-CN, **6:** R= 4-$CH(CH_3)_2$

Scheme 1. The synthesis of the compounds (**1–6**).

Table 1. Cytotoxicity of compounds **1–6** against A549 cell line.

	Concentration (μg/mL)								
Compound	3.9	7.8	15.6	31.2	62.5	125	250	500	IC_{50} (μg/mL)[a]
1	87.4±6.0	83.1±3.2	85.8±8.5	81.8±4.9	67.3±23.5*	34.3±8.9*	33.8±1.5*	25.5±2.9*	86.7±2.9
2	88.9±9.2	81.0±3.4*	82.2±4.0*	79.9±2.1*	41.35±5.5*	29.6±2.7*	28.3±2.6*	24.6±1.5*	53.3±5.8
3	111.5±5.0	105.7±7.6	99.6±7.3	97.4±6.8	94.1±6.1	87.3±2.6	86.4±3.3	84.6±6.7	>500
4	96.0±8.1	90.8±1.8	89.2±16.7	82.1±5.8	73.6±4.8*	57.4±2.8*	46.8±1.9*	31.0±3.1*	193.3±40.4
5	111.4±11.4	87.1±8.8	81.8±10.9	74.9±8.3	69.6±8.6	60.2±3.4	59.4±3.9	57.3±4.7	>500
6	98.8±5.3	80.5±7.4*	73.6±7.7*	64.5±9.1*	55.9±0.8*	59.2±5.9*	55.7±2.2*	47.5±4.1*	435.0±68.7
Mito	81.1±5.3	77.5±6.7*	58.8±17.1*	37.8±3.6*	39.9±4.4*	36.5±5.1*	30.9±5.4*	24.8±1.9*	24.3±2.1

[a]Results are expressed as the mean % of MTT absorbance. Data points represent means of three independent experiments ± SD.
*significantly differences from control $p < 0.05$.

Table 2. Cytotoxicity of compounds **1–6** against C6 cell line.

	Concentration (μg/mL)								
Compound	3.9	7.8	15.6	31.2	62.5	125	250	500	IC_{50} (μg/mL)[a]
1	112.9±11.9	123.7±20.7	121.8±17.7	109.8±8.6	36.4±5.1*	31.4±4.8*	12.7±0.7*	12.3±1.4*	50.7±3.8
2	113.2±13.4	118.9±21.0	111.9±7.5	70.7±15.1	40.7±3.4*	17.7±0.8*	14.7±3.4*	13.2±1.6*	45.0±8.8
3	102.8±5.1	108.2±12.1	97.9±6.6	91.2±1.3	82.7±8.7	64.9±7.8*	59.2±8.9*	62.7±10.6*	>500
4	98.5±11.2	89.5±14.5	104.5±16.3	104.9±11.5	91.6±8.8	66.5±10.6*	48.4±3.2*	42.1±4.7*	216.0±29.5
5	116.2±22.2	120.4±8.6	111.9±16.5	127.9±13.4	102.5±5.1	90.2±8.8	71.5±9.9	41.7±7.0*	415±73.7
6	106.6±3.9	98.7±12.2	111.8±12.6	121.4±23.6	107.6±7.5	83.1±12.1	50.9±5.0*	36.5±5.8*	256.7±40.4
Mito	74.9±8.4*	57.6±3.3*	45.8±8.3*	35.9±3.9*	37.4±7.0*	34.7±4.7*	25.1±4.1*	16.5±2.1*	11.0±1.0

[a]Results are expressed as the mean % of MTT absorbance. Data points represent means of three independent experiments ± SD.
*significantly differences from control $p < 0.05$.

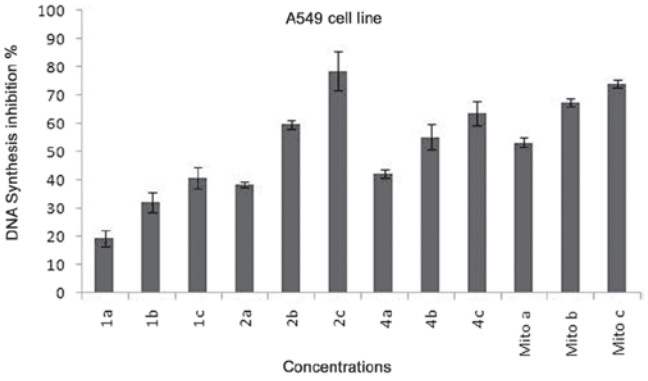


Figure 1. DNA Synthesis inhibitory activity of compounds **1**, **2**, **4** and mitoxantrone on A549 cells. Mean percent absorbance of untreated control cells were assumed 0%. Three different concentrations (**1a** 31.2 μg/mL; **1b** 62.5 μg/mL; **1c** 125μg/mL; **2a** 31.2 μg/mL; **2b** 53.3 μg/mL; **2c** 125 μg/mL; **4a** 62.5 μg/mL; **4b** 125 μg/mL; **4c** 250 μg/mL; **Mito a** 7.8 μg/mL; **Mito b** 24.3 μg/mL; **Mito c** 31.2 μg/mL) of test compounds and mitoxantrone were given. Data points represent means for two independent experiments ± SD of four independent wells. $p < 0.05$.

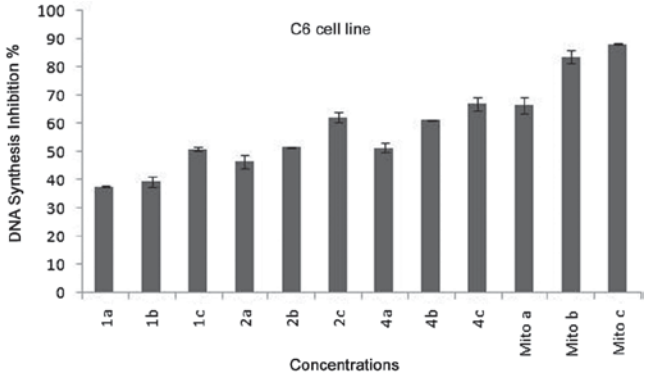


Figure 2. DNA Synthesis inhibitory activity of compounds **1**, **2**, **4** and mitoxantrone on C6 cells. Mean percent absorbance of untreated control cells were assumed 0%. Three different concentrations (**1a** 31.2 μg/mL; **1b** 50 μg/mL; **1c** 125 μg/mL; **2a** 31.2 μg/mL; **2b** 45μg/mL; **2c** 125 μg/mL; **4a** 125 μg/mL; **4b** 216 μg/mL; **4c** 250 μg/mL; **Mito a** 3.9 μg/mL; **Mito b** 11 μg/mL; **Mito c** 15.6 μg/mL) of test compounds and mitoxantrone were given. Data points represent means for two independent experiments ± SD of four independent wells. $p < 0.05$.

was carried out on A549 and C6 cell lines for compounds **1**, **2** and **4** which exhibited significant cytotoxic activity in MTT assay. Afterwards, compounds **1**, **2** and **4** were also subjected to caspase-3 activation assay to determine the possible mechanism of action. Finally, cellular and nuclear morphological changes of C6 cells following exposure to various concentrations of compounds **1**, **2**, **4** and mitoxantrone were also observed.

In MTT assay, cytotoxicity of compounds **1**, **2** and **4** was closer to that of mitoxantrone than other compounds and the most effective cytotoxic agent against two cancer cell lines was found as compound **2** followed by compounds **1** and **4** (Tables 1 and 2). Compound **2** exhibited anticancer activity against A549 and C6 cell lines with IC_{50} values of 53.3 ± 5.8 and 45.0 ± 8.8 μg/mL, whereas mitoxantrone exhibited anticancer activity against both

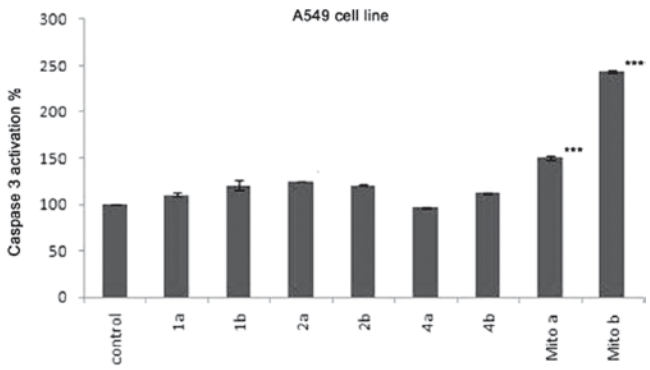


Figure 3. Effect of Ac-DEVD-amc on the activity of caspase-3 induced by compounds **1**, **2**, **4** and mitoxantrone. A546 cells were maintained in cultures for 24 h and then exposed to Ac-DEVD-amc (1.0 mM) 30 min before exposure to **1a** 31.2 μg/mL; **1b** 86.7 μg/mL; **2a** 31,2 μg/mL; **2b** 53.3 μg/mL; **4a** 62.5 μg/mL; **4b** 193.3 μg/mL; **Mito a** 7.8 μg/mL; **Mito b** 24.3 μg/mL). Values represent mean ± S.D. from duplicate samples for each experiment. ***significantly different from respective control cells ($p < 0.001$).

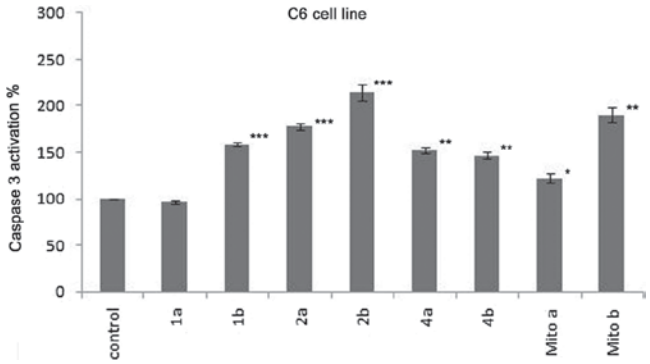


Figure 4. Effect of Ac-DEVD-amc on the activity of caspase-3 induced by compounds **1**, **2**, **4** and mitoxantrone. C6 cells were maintained in cultures for 24 h and then exposed to Ac-DEVD-amc (1.0 mM) 30 min before exposure to (**1a** 31.2 μg/mL; **1b** 50.7 μg/mL; **2a** 31.2 μg/mL; **2b** 45 μg/mL; **4a** 125 μg/mL; **4b** 216 μg/mL; **Mito a** 3.9 μg/mL; **Mito b** 11 μg/mL). Values represent mean ± S.D. from dublicate samples for each experiment.*: significantly different from respective control cells ($p < 0.05$).**significantly different from respective control cells ($p < 0.01$).***significantly different from respective control cells ($p < 0.001$).

of the cancer cell lines with IC_{50} values of 24.3 ± 2.1 μg/mL and 11.0 ± 1.0 μg/mL, respectively. Compounds **1** and **4** exhibited cytotoxic activity against A549 cell line with IC_{50} values of 86.7 ± 2.9 and 193.3 ± 40.4 μg/mL, respectively. IC_{50} values of compounds **1** and **4** against C6 cell line were also determined as 50.7 ± 3.8 and 216.0 ± 29.5 μg/mL, respectively. Cytotoxicity of compound **3** was lower than compound **6**. Cytotoxicity of compound **5** was also similar to compound **3**. These results suggest that 4-methyl, 4-bromo and 3,4-methylenedioxy moieties contribute to the increase in cytotoxicity. On the other hand, low cytotoxicity of compounds **3**, **5** and **6** can be attributed to 4-hydroxy, 4-cyano and 4-isopropyl moieties.

DNA synthesis inhibition activity of compounds **1**, **2** and **4** was evaluated for 24 h. A549 and C6 cell lines were incubated with three different concentrations of compounds that were determined according to their IC_{50}

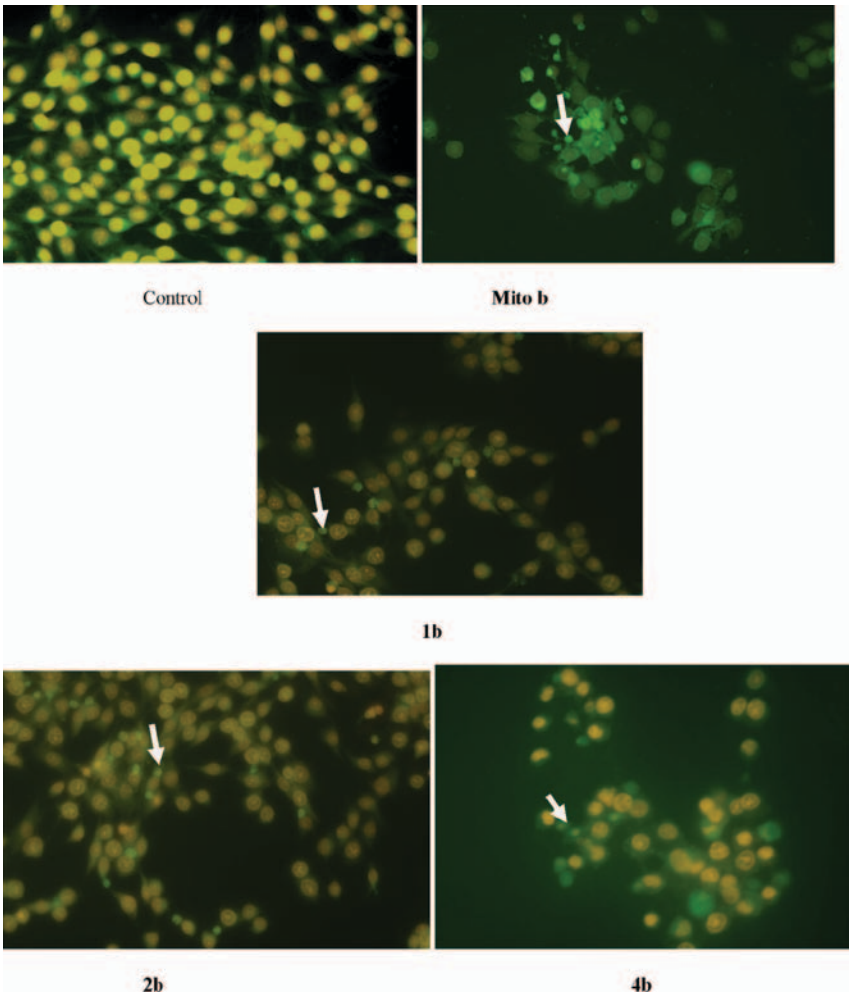


Figure 5. Cellular and nuclear morphological changes of C6 cells following exposure to various concentrations of compound **1**, **2**, **4** and mitoxantrone for 24 h. (**1b** 50 μg/mL; **2b** 45 μg/mL **4b** 216 μg/mL; **Mito b** 11 μg/mL). Cells were distinguished according to the fluorescence emission and the morphological aspect of chromatin condensation in the stained nuclei. Viable cells have uniform bright green nuclei with organized structure. Early apoptotic cells have green nuclei, but perinuclear chromatin condensation is visible as bright green patches or fragments. White arrows indicate apoptotic cells. (See colour version of this figure online at www.informahealthcare.com/enz)

values. Mitoxantrone was used as positive control. The tested compounds showed inhibitory activity in dose-dependent manner.

DNA synthesis inhibitory activity of the compounds on A549 and C6 cell lines is presented in Figures 1 and 2. These results show that compounds tested in this assay cause DNA synthesis inhibition. Among these compounds, the most potent inhibitor of DNA synthesis against A549 and C6 cell lines was found as compound **2**.

Caspase-3 activation, which plays a pivotal role in initiation of cellular events during the early apoptotic process, can be used in cellular assays as a hallmark of apoptosis[25]. A549 and C6 cell lines were incubated with the compounds at two different concentrations. IC_{50} and lower concentrations were preferred for this purpose. As seen in Figure 3, A549 cells treated with compounds **1**, **2** and **4** had no significant differences in caspase-3 activation when compared with controls. This result may be explained by different death mechanism in A549 cells caused by compounds other than apoptosis. On the other hand, these compounds showed significantly different increases in caspase-3 activation on C6 cells when compared with controls (Figure 4). Compound **2** showed the highest activity in caspase-3 activation assay.

Morphological evaluation of early apoptosis in C6 cells was carried out with acridine orange/ethidium bromide staining method due to the fact that only C6 cells displayed early apoptotic response to these compounds. C6 cell lines were incubated with compounds **1**, **2**, **4** and mitoxantrone at IC_{50} concentrations. When incubation period (24h) was over, C6 cells were stained with acridine orange/ethidium bromide and cellular and nuclear morphological changes were monitored in fluorescence microscope (Figure 5). Viable cells had uniform bright green nuclei with organized structure. But early apoptotic cells caused by compounds **1**, **2** and **4** had green nuclei and perinuclear chromatin condensation.

Conclusion

In the present paper, we synthesized a series of pyrazoline derivatives and evaluated their anticancer activity against

A549 and C6 cancer cell lines by means of MTT, DNA synthesis inhibition and caspase-3 activation assays. Among these derivatives, compounds **1**, **2** and **4** exhibited dose-dependent anticancer activity against A549 and C6 cancer cell lines. Although these three compounds showed substantial cytotoxicity and caspase-3 activation in C6 cells, they did not show any early apoptotic effects on A549 cells. Hence, anticancer effects of these compounds may result from different death mechanism in A549 and C6 cell lines.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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