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Cytotoxic and apoptosis inducing effect of some pyrano [3, 2-c] pyridine derivatives against MCF-7 breast cancer cells

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Anti-cancer activities of some pyrano-pyridines have been previously reported. Herein, we investigated anti-proliferative and apoptotic effects of the novel pyrano [3, 2-c] pyridine (P.P, TPM.P, 4-CP.P and 3-NP.P) compounds against MCF-7 breast cancer cells. The MCF-7 cells were cultured in the presence of various concentrations (20–200 μM) of the tested compounds for 3 days and the cell viability was determined by MTT assay. Induction of apoptosis was qualitatively assayed by acridine orange/ethidium bromide (AO/EtBr) staining, DNA fragmentation assay, as well as quantitatively by Annexin V/PI double staining and cell cycle analysis. These compounds inhibited growth and proliferation of the MCF-7 cells in a dose- and time-dependent manner. The IC50 values of P.P, TPM.P, 4-CP.P and 3-NP.P after 24 h of exposure were calculated to be 100±5.0, 180±6.0, 60±4.0 and 140±5.0 μM, respectively. 4-CP.P was determined as the most potent compound and was chosen for further studies. The result of flow cytometric cell cycle analysis indicated an increase in sub-G1 population after 72 h treatment of the cells. Furthermore, this was accompanied by exposure of phosphatidylserine (PS) in the outer cell membrane after time course of treatment with the 4-CP.P. Based on these observations, the pyrano [3, 2-c] pyridines can be regarded as a valuable candidate for further pharmaceutical evaluations.

Keywords: apoptosis, pyrano-pyridine, breast cancer, MCF-7 cells

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Abbreviations: P.P, 2-amino-8-benzylidene-5,6,7,8-tetrahydro-6-phenethyl-4-phenyl-4h-pyrano-[3,2,c] pyridine-3-carbonitrile; TPM.P, 2-amino-5,6,7,8-tetrahydro-6-phenethyl-4-thiophen-2-yl-8-(thiophen-2-yl)methylene) 4h-pyrano-[3,2-c]pyridine-3-carbonitrile; 4-CP.P, 8-(4-chlorobenzylidene)-2-amino-4-(4-chlorophenyl)-5,6,7,8-tetrahydro-6-phenethyl-4h-pyrano-[3,2-c] pyridine-3-carbonitrile; 3-NP.P, 8-(3-nitrobenzylidene)-2-amino-5,6,7,8-tetrahydro-4-(3-nitrophenyl)-6-phenethyl-4h-pyrano[3,2-c]pyridine-3-carbonitrile; FBS, fetal bovine serum; MTT, 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; PI, propidium iodide; AO/EtBr, Acridine orange /ethidium bromide

INTRODUCTION

Breast cancer is the most prevalent cancer among women and one of the main reasons of cancer deaths in women worldwide (Chen *et al*., 2013). More than a million persons have been diagnosed with the illness in 2008 and annually more than 200000 new cases of breast cancer are identified (Germano & O'Driscoll, 2009). Extensive research has been done in conventional therapies such as chemotherapy, surgery and radiotherapy. Even though chemotherapy has been widely respected to treat breast cancer (Huang *et al*., 2012) , the chemotherapeutic agents like anthracyclines bring very serious side effects. Also, their effectiveness is limited due to drug resistance (Huang *et al*., 2010; Lage, 2003). Therefore, it is essential to develop safe and effective novel alternative agents for treatment of this disease. Previous studies have shown that some chemical compounds are excellent resources of effective agents for treatment of many diverse cancers via induction of apoptosis (Cragg & Newman, 2005; Haefner, 2003). Apoptosis, as a physiological event, refers to programmed cell death distinguished by DNA fragmentation, cellular shrinking, chromatin accumulation and making of apoptotic bodies (Taylor *et al*., 2008).

Pyrano [3, 2-c] pyridines and their derivatives have been found to possess many interesting biological properties, including anti-cancer (El-Subbagh *et al*., 2000; Magedov *et al*., 2008), anti-bacterial (Hanawa *et al*., 2004; Kumar & Rajendran, 2004), anti-fungal and anti-algal (Cantrell *et al*., 2005), anti-inflammatory and anti-malaria (Isaka *et al*., 2001) activities. Additionally, they have demonstrated to be effective in treatment of human cancer cells (Magedov *et al*., 2007).

The purpose of the research presented here was to investigate the effect of four novel compounds derived from pyrano [3, 2-c] pyridines (Fig. 1) on growth rate and viability of the MCF-7 cells. Moreover, in this cell line induction of apoptosis was also evaluated for the most active compound among those tested here.

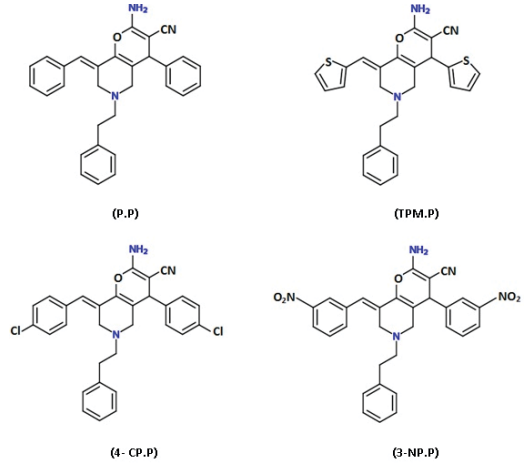
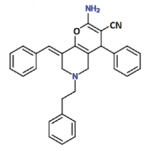
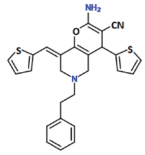
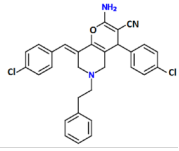
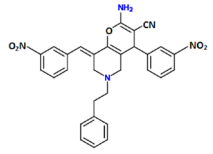


Figure 1. Chemical structure of the investigated pyrano-pyridine compounds.

Table 1. Effect of the new pyrano [3, 2-c] pyridine derivatives on the MCF-7 cell viability.
Cells were treated with various concentrations of the compounds for 24, 48 and 72 hrs. Cell viability was evaluated by MTT assay. Data were expressed as a percentage of control assayed in the absence of the compounds. Each point represents the mean ± S.D. of three independent experiments ($p<0.05$).

Compound Name	Concentration (μM)	Viability (% of Control)		
		24h	48 h	72h
P.P 	0	100	100	100
	20	93±1.8	88±1.9	76±0.8
	40	88±3.7	75±3.2	54±2.8
	60	74±0.9	56±2.2	36±2.6
	80	61±1.1	40±2.8	27±1.7
	100	48±3.7	31±0.9	20±1.8
	120	36±3.1	22±1.9	13±3.4
	140	26±2.6	16±1.8	11±2.7
	160	21±0.9	13±3.3	9±1.50
	200	19±2.6	10±1.7	6±1.20
TPM.P 	0	100	100	100
	20	95±2.6	91±0.2	86±2.5
	40	89±0.7	87±1.2	82±3.5
	60	83±3.5	81±2.2	71±4.2
	80	76±1.5	71±1.6	56±1.3
	100	69±1.3	62±1.4	46±1.7
	120	63±0.8	56±3.4	41±1.2
	140	59±3.3	51±4.1	33±2.1
	160	55±3.1	45±2.8	28±1.6
	200	47±2.7	37±3.2	19±3.1
4-CP.P 	0	100	100	100
	20	66±1.1	56±3.9	51±2.6
	40	56±3.9	50±0.5	39±1.7
	60	50±1.5	45±3.8	32±3.9
	80	47±2.4	43±0.6	31±0.7
	100	42±1.2	35±2.5	29±2.1
3-NP.P 	0	100	100	100
	20	92±2.4	89±1.2	82±2.0
	40	90±1.6	84±3.3	73±2.2
	60	85±3.2	79±1.3	67±2.4
	80	77±1.9	72±1.9	56±2.1
	100	69±3.9	62±0.9	47±1.6
	120	58±1.1	51±2.9	41±2.5
	140	52±3.7	44±0.8	37±3.1
	160	45±2.3	37±2.3	29±1.8
	200	38±3.1	25±2.4	18±2.3

METHODS AND MATERIALS

Materials. Fetal bovine serum (FBS), penicillin-streptomycin and cell culture medium (RPMI-1640) were obtained from Gibco BRL Life Technologies (Paisley, Scotland). Culture plates were acquired from SPL (Korea). MTT reagent [3 0-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] and dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich (Germany). Annexin-V/PI (propidium iodide) staining kit was acquired from Roche Corporation (Germany). The MCF-7 cells were acquired from the Pasture Research Institute (Iran).

Preparation of the investigated 4H-pyrano [3,2-c] pyridine derivatives. 2-Amino-4-aryl-8-[(E)-arylmethylidene]-5,6,7,8-4Hpyrano [3,2-c] pyridine derivatives were synthesized at a peptide chemistry research center, K. N. Toosi University of Technology. Briefly, reaction of 3, 5-bis [(E)-arylmethylidene]-tetrahydro-4(1H)-pyridinones with malononitril in aqueous media and in the presence of diammonium hydrogen phosphate (10%) or piperidine (10%) leads to pyranopyridines at room temperature. The structures of the target compounds, including 2-amino-8-benzylidene-5,6,7,8-tetrahydro-6-phenethyl-4-phenyl-4h-pyrano-[3,2,c] pyridine-3-carbonitrile (P.P), 2-amino-5,6,7,8-tetrahydro-6-phenethyl-4-thiophen-2-yl-8-(thiophen-2-yl)methylene) 4h-pyrano-[3,2-c]pyridine-3-carbonitrile (TPM.P), 8-(4-chlorobenzylidene)-2-amino-4-(4-chlorophenyl)-5,6,7,8-tetrahydro-6-phenethyl-4h-pyrano-[3,2-c] pyridine-3-carbonitrile (4-CP.P) and 8-(3-nitrobenzylidene)-2-amino-5,6,7,8-tetrahydro-4-(3-nitrophenyl)-6-phenethyl-4H-pyrano[3,2-c]pyridine-3-carbonitrile (3-NP.P) were established by IR, ^{1}H-NMR, C-NMR, as well as mass spectrometry and elemental analysis (Balalaie *et al*., 2013).

Cell culture conditions. MCF-7 cells were cultivated in RPMI 1640 medium enriched with 10% FBS (V/V), 100 μg/ml streptomycin and 100 U/ml penicillin at 37°C in a humidified atmosphere of 5% CO_2 (Hassan *et al*., 2013).

Cytotoxicity analysis. Viability of the cells was analyzed with the MTT colorimetric test that is able to detect viable cells *via* reduction of the yellow tetrazolium salt to purple formazan. Briefly, the MDF-7 cells (6×10^3 cells/well) were seeded into 96-well plates in triplicate and cultured in the course of the night. The cells were treated with various concentrations (20 to 200 μM) of the compounds for various times (24 h, 48 h and 72 h) and maintained in a CO_2 incubator. At the end of each treatment, the medium was eliminated from the wells and a fresh medium including MTT reagent (final concentration of 0.5 mg/mL) was added to each well. Then, the plates were maintained at 37°C for 4 h. Next, the medium was eliminated from the wells and the formazan blue crystals were dissolved by adding 200 μl of DMSO. Subsequently, cell viability and IC50 (a concentration causing 50% reduction in the viable cell number) was

determined by measuring the absorbance at 570 nm using a microplate reader (Expert 96, Asys Hitech) (Mahdavi *et al*., 2011). Data was obtained from at least three separate experiments, where untreated and DMSO treated cells served as a negative and positive control, respectively.

Cellular morphology study. To confirm the phenomenon of apoptosis, acridine orange (AO) and ethidium bromide (EtBr) double staining test was performed. Into 24-well plates, 6×10^3 cells/well were seeded and treated with the most active compound (4-CP.P) at indicated concentrations (IC50 value) for 72 h. Cell morphology was assessed by a phase-contrast microscope (Zeiss, Germany) and cell death was studied morphologically by using fluorescent dyes (AO/EtBr). In brief, the cells were collected by centrifugation and resuspended in 100 ml of cold PBS. Then, 20 ml of staining solution (1:1) mixture of 100 mg/ml of EtBr and 100 mg/ml of AO (Sigma Chemical Co.) was added. Afterwards, the morphology of cells was analyzed by ultraviolet fluorescence microscopy (Olympus BX41, Germany) (Czyz *et al*., 2008).

DNA fragmentation assay. MCF-7 cells were treated at only one dosage (at IC50 Value) of the 4-CP.P for different time intervals. The cells were collected after 48 and 72 h, washed with PBS and then incubated in 1 ml of lysis buffer [0.01M Tris-HCl (pH 8.0), 0.1 M NaCl, 0.025 M EDTA (pH 8.0), and 1% SDS] which contained 10 µg/mL of RNase A, for 1 h at 37°C, followed by treatment with 0.2 mg/mL proteinase K for 2 h at 50°C. After centrifugation at 12000×*g*, the DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) to eliminate the lipids and proteins. Then, DNA in the supernatant was precipitated overnight in ethanol at 20°C and pelleted at 15000×*g*. The sample of dry pellets were resuspended in TE buffer (10 mM Tris-HCl, pH 7.9, 1 mM EDTA) and loaded onto a 2% agarose gel containing ethidium bromide, and then electrophoresed for 2 h (Aryapour *et al*., 2012).

Cell cycle analysis. Cells (1×10^6) were seeded in 24 well plates and following 24 h incubation were treated with the active compound (4-CP.P) for 72 h. Then, the cells were detached by employing a trypsin-EDTA solution at 37°C for 5 min. Afterwards, the trypsin activity was stopped by adding 10% FBS–RPMI 1640 medium. Then, the cells were collected, placed in a microtube and centrifuged at 1000 rpm for 5 min. Next, the cells were washed with PBS buffer and centrifuged at 1000 rpm for 5 min. Subsequently, they were fixed in cold 70% ethanol, incubated for 30 min at 4°C. Then, the cells were resuspended in 1 ml of PBS, treated with ribonuclease (50 µl of a 100 µg/ml stock of RNase) for 30 min at 37°C and 200 µl of PI (from 50 µg/ml stock solution) were added. The percentage of cells calculated to be in the sub-G1, G0/G1, S, and G2/M phases was analyzed by flow cytometry (BD FACSCalibur flow cytometer, USA) (Hu *et al*., 2002).

Apoptotic cell quantification by flow cytometry. The cells undergoing apoptosis were identified by binding of the annexin V protein to the exposed phosphatidylserine (PS) residues at cell surface (Riccardi & Nicoletti, 2006). In this assay, an annexin V-FITC/ propidium iodide (PI) double staining method was used according to the manufacturer's protocol. Briefly, after double washing of the treated and untreated cells with PBS, 1×10^6 cells were resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Then, 5 µl of annexin V-FITC and 5 µl PI were added. The mixture was incubated for 15 min in the dark at room temperature and then analyzed by flow cytometry (BD FACSCalibur flow cytometer, USA) with emission filters of 515–545 nm for FITC (green) and 600 nm for PI (red). A total of 10000 cells per sample were acquired, and the data were analyzed with Cell Quest software (Becton Dickinson, San Jose, USA) (Saadat *et al*., 2015).

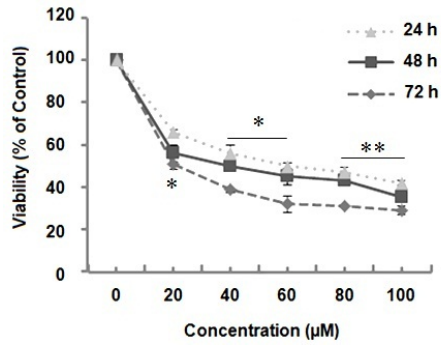


Figure 2. Anti-proliferative effects of 4-CP.P on the MCF-7 cells. The cells were treated with different concentrations (20–100 µM) of 4-CP.P for 24–72 h. Cell viability was evaluated by MTT assay. Results are mean values ± S.D. of three independent experiments. (*$p<0.05$, **$p<0.01$).

Statistical assessment. Data are presented as the mean ± S.D. of three separate experiments and were statistically analyzed using Student T-test with Excel software. Comparison between groups was also confirmed by one-way analysis of variance (ANOVA) followed by a specific post-hoc test to analyze the difference. Differences were considered significant at $p<0.05$.

RESULTS

Previous studies showed that some pyrano [3, 2-c] pyridone derivatives have inhibitory effects on growth of different cancer cell lines (Magedov *et al*., 2007; Magedov *et al*., 2008). In this study, we examined inhibition of the MCF-7 cells proliferation of the novel pyrano [3, 2-c] pyridines. These compounds inhibited the growth of MCF-7 cells in a time and dose dependent manner (Table 1). As shown in Table 1, after 24 h exposure of the cells, the IC50 values for P.P, TPM.P, 4-CP.P and 3-NP.P were calculated to be 100±5.0, 180±6.0, 60 ±4.0 and 140±5.0 µM, respectively. 4-CP.P with the IC50 values of 60 µM, 40 µM and 20 µM for 24 h, 48 h and 72 h respectively, was the most active compound against MCF-7 cells (Fig. 2, Table 2). Based on these data, we selected 4-CP.P with IC50 value of 60 µM (at 24 h) for further studies.

Under a phase-contrast microscope, the control cells were observed in a completely crowded and attached

Table 2. The IC50 values of the investigated compounds after 24–72 h.
4-CP.P was more active in comparison with other compounds, and the IC50 value of this compound for MCF-7 cells was calculated to be 60±4.0, 40±7.0 and 20±3.0 µM after 24, 48 and 72 hrs, respectively. Each value represents the average of triplicate measurements ± S.D.

Compound	IC50 (µM)		
	24 h	48 h	72 h
P.P	100±5.0	70±3.0	40±4.0
TPM.P	180±6.0	140±4.0	90±3.0
4-CP.P	60±4.0	40±7.0	20±3.0
3-NP.P	140±5.0	120±6.0	100±5.0

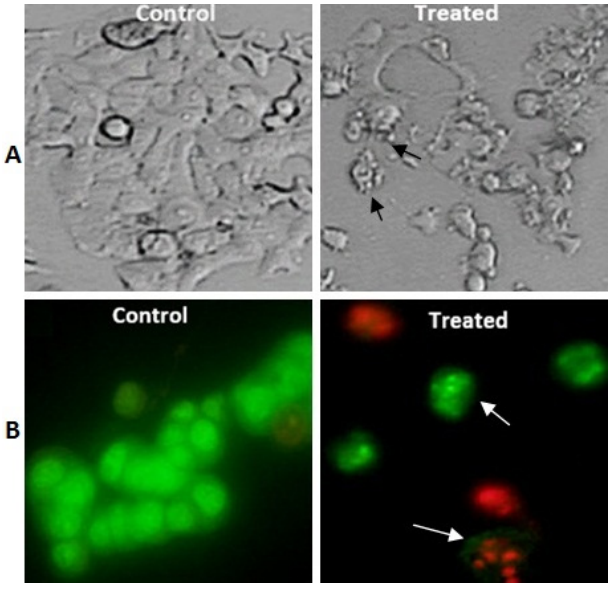


Figure 3. Phase-contrast and fluorescence microscopic observation of the MCF-7 cells treated with 4-CP.P (at IC50 value) for 72 h.
(**A**) Phase-contrast image of the MCF-7 cells. The control cells have completely crowded and attached, while the treated cells obviously have been detached, condensed and fragmented. (**B**) fluorescence microscopy evaluation of the treated and untreated cells after AO/EB staining. The control cells had large and round nucleus, without any condensation or fragmentation of the chromatin, whereas the cells treated with 4-CP.P showed high percentages of cell shrinkage, chromatin fragmentation, and apoptotic bodies, a typical morphological feature of apoptosis. Apoptotic bodies are indicated by arrows. Magnification was 200×.

form to the culture plate, while the treated cells obviously were condensed, fragmented and then detached from the culture plate (Fig. 3A). To evaluate apoptosis, the MCF-7 cells were treated with the indicated concentration (IC50 values) of 4-CP.P. Then, the morphology changes were inspected by AO/EtBr dual staining. The

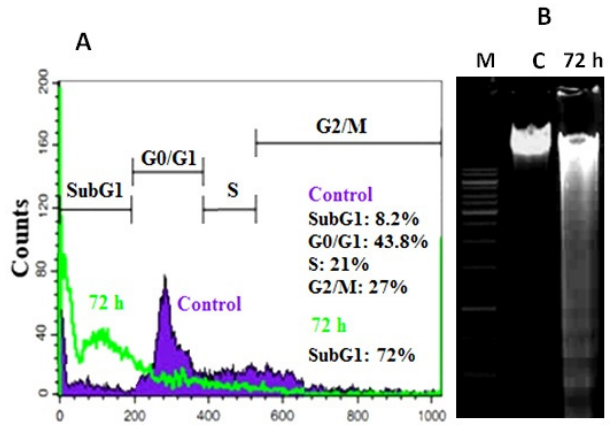


Figure 4. Effect of 4-CP.P on cell cycle phase distribution of the MCF-7 cells.
(**A**) Representative histograms of DNA content in the cells treated with 4-CP.P (60 μM) for 72 h. Horizontal and vertical axes indicate the relative nuclear DNA content and number of cells, respectively. Percentage of the sub-G1 phase cells was increased in the treated cells after 72 h of treatment as compared to untreated control. Significant difference from the control at the same phase is indicated as $p<0.05$. (**B**) The DNA fragmentation analysis following treatment of the MCF-7 cells with 4-CP.P. C, control cells; M, DNA Marker; 72 h, cells treated with 60 μM of 4-CP.P for 72 h.

results revealed that the 4-CP.P is an apoptosis inducing agent in the MCF-7 cells (Fig. 3B). The images taken under fluorescence microscope showed that the control cells had large and round nucleus without any condensation or fragmentation of the chromatin. However, cell shrinkage, nucleus condensation, chromatin fragmentation, as well as formation of apoptotic bodies as a typical morphological feature of apoptosis, have been observed 72 h after treatment of the cells with 60 μM 4-CP.P (Fig. 3B). DNA fragmentation assay was performed with agarose gel electrophoresis to confirm morphological changes in the 4-CP.P treated cells (Fig. 4B).

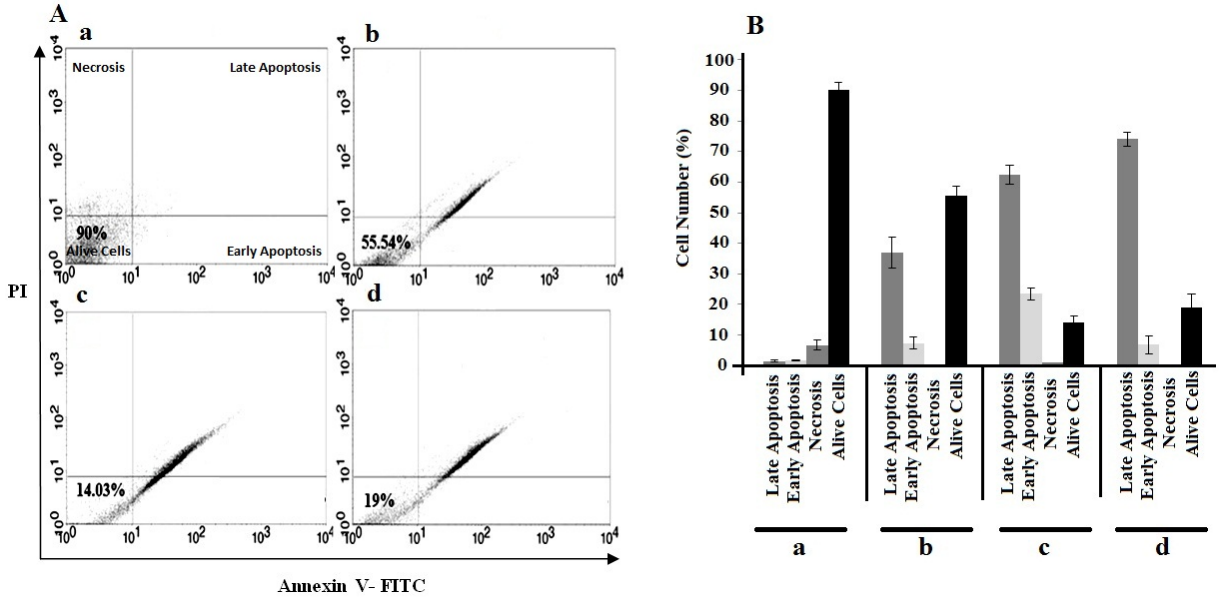


Figure 5. Quantitative analysis of apoptotic cells induced by 4-CP.P using annexin V/PI double staining assay.
(**A**) Representative flow cytometry results of the apoptotic cells after 24–72 h treatment. (a) Untreated cells, (b) cells treated for 24 h, (c) cells treated for 48 h, (d) cells treated for 72 h. As is evident from the figure, a shift was accreted to late apoptosis or necrosis (upper-right quadrant panel), as compared to control. (**B**) The percentage of live, necrotic, early apoptotic and late apoptotic cells. The results are expressed as mean ± S.D. of three independent experiments. $p<0.05$.

The data revealed that the 4-CP.P induces apoptosis in the MCF-7 cells.

In order to gain an insight into the mode of cell death mechanism by 4-CP.P, the cell cycle phase distribution was analyzed by means of Fluorescence Activated Cell Sorting (FACS) flow cytometer. The FACS data described the effect of indicated concentration of 4-CP.P (60 μM) on cell cycle phase distribution of DNA. Our results demonstrated that the DNA content of sub G1 phase cells was increased in the treated cells after 72 h of treatment as compared to the untreated cells. According to our data, the proportion of the cells in the sub-G1 phase of control cells was observed at 8.2%, while sub-G1 apoptotic cell percentage increased to 72% after 72 h treatment (Fig. 4A). These findings suggested that 4-CP.P induces sub-G1 cell cycle arrest and induction of apoptosis in MCF-7 cells.

Our previous data showed that 4-CP.P induced apoptosis in the MCF-7 cells. To confirm the previous results, the percentage of apoptotic cells was assessed by annexin V-FITC/PI staining (Fig. 5). We investigated redistribution of the plasma membrane of the cells (as a hallmark of apoptosis) to visible phosphatidyl serine after double staining with annexin-V/PI. In this assay, the MCF-7 cells that were single-positive stained with annexin V-FITC were considered as mostly early apoptotic cells, and the cells that were single positive stained with PI were considered as mostly necrotic cells, while the cells that were double-positive stained could be either necrotic or late apoptotic cells. The results indicated that after 24, 48 and 72 h treatment of the cells with 4-CP.P (at IC50 value), the percentage of apoptotic cells was 37%, 62% and 74%, respectively (Fig. 5). These data showed that 4-CP.P could effectively induce apoptosis in MCF-7 cells in a time- dependent manner.

DISCUSSION

Breast cancer is a heterogeneous disease in which certain cells in the breast become abnormal and multiply uncontrollably to form a tumor (Kravchenko *et al*., 2011).

This disease is often (but not always) caused by a type of carcinoma called adenocarcinoma which originates in the glandular tissue. The MCF-7 cells are human breast cancer cells that are used widely for studies of tumor biology (Holliday & Speirs, 2011). Many synthetic chemical compounds have been used to treat the MCF-7 human breast cancer. The study presented here was carried out to evaluate the cytotoxic potential of the novel pyrano-pyridine derivatives on the MCF-7 cell line. Furthermore, the most active compound was chosen and used for further experiments such as: DNA fragmentation assay, cell cycle analysis and annexin V/PI double staining assay. These derivatives have been reported to be cytotoxic for several types of cancer cells (Magedov *et al*., 2007). For example, cytotoxic activity of the several thieno [2, 3-b] pyridine derivatives against MCF-7 and HepG-2 cell lines has been reported (Hassan *et al*., 2013). In another study, cytotoxic activity of several 1,4-dihydropyridine (DHP) compounds containing nitroimidazole moiety at the C4 position were evaluated against four different cancer cell lines (Magedov *et al*., 2007). Our compounds were developed by substituting different groups, such as phenyl (P.P), thiophene (TPM.P), 4-chlorophenyl (4-CP.P) and 3-nitrophenyl (3-NP.P) at the 4-position of the phenyl ring (Table 1). Substitution of the 4-chloro phenyl ring (4-CP.P) with phenyl, thiophene, and also 3-nitrophenyl groups, increases its activity which suggests the presence of an electron-withdrawing group (Cl) at the 4-position, but not at the 3-position (NO_2) of phenyl, will lead to a remarkable increase in the cytotoxic effects of these compounds. However, presence of another electron-withdrawing group, such as NO_2 (3-NP.P) at the 3-position, reduces the activity of the compound (Table 1 and Table 2). This suggests that electron-withdrawing groups at the 4-position of the phenyl ring can be effective for increasing the cytotoxic activity of the tested compounds. Over all, the 3-nitro (3-NP.P) and other analogues (P.P, TPM.P) were significantly less potent than 4-CP.P (Table 1). In addition, it was observed that the most active derivative (4-CP.P) could inhibit cell viability and proliferation through promotion of apoptosis. 4-CP.P caused a reduction in cell viability in a dose and time dependent manner (Table 1, Fig. 2). AO/EtBr staining (Fig. 3B), sub-G1 cell cycle arrest (Fig. 4A) and annexin V/PI double staining (Fig. 5) revealed that the treated cells undergo apoptosis. Cell cycle analysis revealed that the decrease in proliferation was associated with the sub-G1 phase arrest (Fig. 4A). Indeed, the increase in the population of sub-G1 cells was accompanied with a decrease in G0/G1, S and G2/M cells after 72 h. In parallel to the sub-G1 cell cycle arrest, apoptosis induction was observed in the MCF-7 cells after 4-CP.P treatment (Fig. 4B, 5). These data suggested that the breakdown of DNA that resulted in cell killing could be due to the cell cycle arrest and apoptosis induction (Rahimi *et al*., 2015; Dehkordi *et al*., 2015). These findings correspond with recent studies that showed the cytotoxicity and apoptosis inducing effects of different types of pyrano-pyridines on various types of cancer cells, such as human liver cancer cells, human cervical cancer cells and Human T-cell leukemia cells (Magedov *et al*., 2007; Magedov *et al*., 2008; Hassan *et al*., 2013).

In summary, our study demonstrated that the novel derivatives of pyrano-pyridines exhibit an anti-proliferative effect on the human breast cancer MCF-7 cells. According to our data, it was shown that 4-CP.P is the most active compound in comparison with the other compounds. This compound inhibited proliferation of the MCF-7 cells and was associated with induction of apoptosis. Overall, this compound (4-CP.P) can be regarded as a valuable candidate for further pharmaceutical evaluations. However, further experiments are needed to improve anti-proliferative properties of these compounds in the future.

Conflict of interest

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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REFERENCES

Aryapour H, Mahdavi M, Mohebbi SR, Zali MR, Foroumadi A (2012) Anti-proliferative and apoptotic effects of the derivatives from 4-aryl-4H-chromene family on human leukemia K562 cells. *Arch Pharmacal Res* **35**: 1573–1582. https://doi.org/10.1007/s12272-012-0908-y

Balalaie S, Mehrazar M, Haghighatnia Y (2013) Efficient synthesis of 2-amino-4-aryl-8-[(E)-arylmethylidene]-5, 6, 7, 8-4 H pyrano [3, 2-c]

pyridine in green media. *Green Chem Lett Rev* **6**: 101–105. https://doi.org/10.1080/17518253.2012.709280
Cantrell C, Schrader K, Mamonov L, Sitpaeva G, Kustova T, Dunbar C, Wedge D (2005) Isolation and identification of antifungal and antialgal alkaloids from Haplophyllum sieversii. *J Agr Food Chemi* **53**: 7741–7748. https://doi.org/10.1021/jf051478v
Chen L, Chen J, Xu H (2013) Sasanquasaponin from *Camellia oleifera* Abel. induces cell cycle arrest and apoptosis in human breast cancer MCF-7 cells. *Fitoterapia* **84**: 123–129. https://doi.org/10.1016/j.fitote.2012.11.009
Cragg GM, Newman DJ (2005) Plants as a source of anti-cancer agents. *J Ethnopharmacol* **100**: 72–79. https://doi.org/10.1016/j.jep.2005.05.011
Czyz M, Jakubowska J, Sztiller-Sikorska M (2008) STI571/doxorubicin concentration-dependent switch for diverse caspase actions in CML cell line K562. *Biochem Pharmacol* **75**: 1761–1773. https://doi.org/10.1016/j.bcp.2008.02.004
Dehkordi MF, Dehghan G, Mahdavi M, Hosseinpour Feizi MA (2015) Multispectral studies of DNA binding, antioxidant and cytotoxic activities of a new pyranochromene derivative. *Spectrochimica Acta – Part A: Mol Biomol Spectr* **145**: 353–359. https://doi.org/10.1016/j.saa.2015.03.026
El-Subbagh HI, Abu-Zaid SM, Mahran MA, Badria FA, Al-Obaid AM (2000) Synthesis and biological evaluation of certain α, β-unsaturated ketones and their corresponding fused pyridines as antiviral and cytotoxic agents. *Journal of medicinal chemistry* **43**: 2915–2921. https://doi.org/10.1021/jm000038m
Germano S, O'Driscoll L (2009) Breast cancer: understanding sensitivity and resistance to chemotherapy and targeted therapies to aid in personalised medicine. *Curr Cancer Drug Targets* **9**: 398–418. https://doi.org/10.2174/156800909788166529
Haefner B (2003) Drugs from the deep: marine natural products as drug candidates. *Drug Discovery Today* **8**: 536–544. https://doi.org/10.1016/S1359-6446(03)02713-2
Hanawa F, Fokialakis N, Skaltsounis A-L (2004) Photo-activated DNA binding and antimicrobial activities of furoquinoline and pyranoquinolone alkaloids from rutaceae. *Planta Medica* **70**: 531–535. https://doi.org/10.1055/s-2004-827153
Hassan A, Sarg M, Said M, El-Sebaey S (2013) Utility of thieno [2, 3-b] pyridine derivatives in the synthesis of some condensed heterocyclic compounds with expected biological activity. *Universal Organic Chem* **1**: 2. https://doi.org/10.7243/2053-7670-1-2
Holliday DL, Speirs V (2011) Choosing the right cell line for breast cancer research. *Breast Cancer Res* **13**: 215. https://doi.org/10.1186/bcr2889
Hu H, Ahn NS, Yang X, Lee YS, Kang KS (2002) Ganoderma lucidum extract induces cell cycle arrest and apoptosis in MCF-7 human breast cancer cell. *Int J Cancer* **102**: 250–253. https://doi.org/10.1002/ijc.10707
Huang K, Huang Y, Diao Y (2012) Wogonin induces apoptosis and down-regulates survivin in human breast cancer MCF-7 cells by modulating PI3K–AKT pathway. *Int Immunopharmacol* **12**: 334–341. https://doi.org/10.1016/j.intimp.2011.12.004
Huang W-J, Wu C-L, Lin C-W, Chi L-L, Chen P-Y, Chiu C-J, Huang C-Y, Chen C-N (2010) Marchantin A, a cyclic bis (bibenzyl ether), isolated from the liverwort *Marchantia emarginata* subsp. tosana induces apoptosis in human MCF-7 breast cancer cells. *Cancer Lett* **291**: 108–119. https://doi.org/10.1016/j.canlet.2009.10.006
Isaka M, Tanticharoen M, Kongsaeree P, Thebtaranonth Y (2001) Structures of Cordypyridones AD, Antimalarial N-Hydroxy-and N-Methoxy-2-pyridones from the Insect Pathogenic Fungus Cordyceps n ipponica. *J Organic Chem* **66**: 4803–4808. https://doi.org/10.1021/jo0100906
Kravchenko J, Akushevich I, Seewaldt VL, Abernethy AP, Lyerly HK (2011) Breast cancer as heterogeneous disease: contributing factors and carcinogenesis mechanisms. *Breast Cancer Res Treatment* **128**: 483–493. https://doi.org/10.1007/s10549-011-1347-z
Kumar NV, Rajendran S (2004) One-pot synthesis and the biological activities of 4-methylpyrano [3, 2-C]-quinolin-2, 5-[6H]-diones. *Asian J Chem* **16**: 1911–1914
Lage H (2003) Drug resistance in breast cancer. *Cancer Therapy* **1**: 81–91
Magedov IV, Manpadi M, Evdokimov NM, Elias EM, Rozhkova E, Ogasawara MA, Bettale JD, Przheval'skii NM, Rogelj S, Kornienko A (2007) Antiproliferative and apoptosis inducing properties of pyrano [3, 2-c] pyridones accessible by a one-step multicomponent synthesis. *Bioorg Med Chem Lett* **17**: 3872–3876. https://doi.org/10.1016/j.bmcl.2007.05.004
Magedov IV, Manpadi M, Ogasawara MA, Dhawan AS, Rogelj S, Van Slambrouck S, Steelant WF, Evdokimov NM, Uglinskii PY, Elias EM (2008) Structural simplification of bioactive natural products with multicomponent synthesis. 2. Antiproliferative and antitubulin activities of pyrano [3, 2-c] pyridones and pyrano [3, 2-c] quinolones. *J Med Chem* **51**: 2561–2570. https://doi.org/10.1021/jm701499n
Mahdavi M, Davoodi J, Zali MR, Foroumadi A (2011) Concomitant activation of caspase-9 and down-regulation of IAP proteins as a mechanism of apoptotic death in HepG2, T47D and HCT-116 cells upon exposure to a derivative from 4-aryl-4H-chromenes family. *Biomed Pharmacotherapy* **65**: 175–182. https://doi.org/10.1016/j.biopha.2011.03.001
Rahimi R, Mahdavi M, Pejman S, Zare P, Balalaei S (2015) Inhibition of cell proliferation and induction of apoptosis in K562 human leukemia cells by the derivative (3-NpC) from dihydropyranochromenes family. *Acta Biochim Pol* **62**: 83–88. https://doi.org/10.18388/abp.2014_825
Riccardi C, Nicoletti I (2006) Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nature Protocols* **1**: 1458–1461. https://doi.org/10.1038/nprot.2006.238
Saadat YR, Saeidi N, Vahed SZ, Barzegari A, Barar J (2015) An update to DNA ladder assay for apoptosis detection. *BioImpacts: BI* **5**: 25–28. https://doi.org/10.15171/bi.2015.01
Taylor RC, Cullen SP, Martin SJ (2008) Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol* **9**: 231–241. https://doi.org/ 10.1038/nrm2312