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

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 200,000 new cases of breast cancer are identified (Germano & O’Driscol, 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.
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).

Table 1. Effect of the new pyrano [3,2-c] pyridine derivatives on the MCF-7 cell viability.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Concentration (µM)</th>
<th>24h Viability (% of Control)</th>
<th>48h Viability (% of Control)</th>
<th>72h Viability (% of Control)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td><strong>P.P</strong></td>
<td></td>
<td>100</td>
<td>93±1.8</td>
<td>88±1.9</td>
</tr>
<tr>
<td><strong>TPM.P</strong></td>
<td></td>
<td>100</td>
<td>89±0.7</td>
<td>87±1.2</td>
</tr>
<tr>
<td><strong>4-CP.P</strong></td>
<td></td>
<td>100</td>
<td>95±2.6</td>
<td>91±0.2</td>
</tr>
<tr>
<td><strong>3-NP.P</strong></td>
<td></td>
<td>100</td>
<td>66±1.1</td>
<td>56±3.9</td>
</tr>
</tbody>
</table>

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-tetrahydro-4-(3-nitrophenyl)-6-phenethyl-4H-pyra

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

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5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

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5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyra-

5,6,7,8-tetrahydro-6-phenethyl-4H-pyr
MCF-7 cells were treated with 0.2 mg/mL proteinase K for 2 h at 50°C. After centrifugation at 12 000×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 15 000×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 (Arayapour et al., 2012).

Cell cycle analysis. Cells (1×10⁶) 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 1500×g 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 phosphatidyserine (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, 1×10⁶ cells were resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂), then, 5µl of annexin V-FITC and 5µl 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 10 000 cells per sample were acquired, and the data were analyzed with Cell Quest software (Becton Dickinson, San Jose, USA) (Saadat et al., 2015).

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; Mage dov 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, 40±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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>24 h (µM)</th>
<th>48 h (µM)</th>
<th>72 h (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.P</td>
<td>100±5.0</td>
<td>70±3.0</td>
<td>40±4.0</td>
</tr>
<tr>
<td>TPM.P</td>
<td>180±6.0</td>
<td>140±4.0</td>
<td>90±3.0</td>
</tr>
<tr>
<td>4-CP.P</td>
<td>60±4.0</td>
<td>40±7.0</td>
<td>20±3.0</td>
</tr>
<tr>
<td>3-NP.P</td>
<td>140±5.0</td>
<td>120±6.0</td>
<td>100±5.0</td>
</tr>
</tbody>
</table>
form to the culture plate, while the treated cells obviously have been detached, condensed and fragmented. (B) fluorescence microscopy evaluation of the treated and untreated cells after AO/EtBr 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×.

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/EtBr 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×.

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 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.
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 cytometry. 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 pyra-no-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 cytoxic 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 (NO2) of phenyl, will lead to a remarkable increase in the cytotoxic effects of these compounds. However, presence of another electron-withdrawing group, such as NO2 (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.

**Acknowledgement**

The authors appreciate support of this investigation by the research council of University of Tabriz, Tabriz, Iran. We would also like to thank Dr. Saeed Balalai for providing the pyrano-pyridine compounds.

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